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Study of Toxicological Evaluation of Fire Suppressants and Extinguishers

NASA CONTRACT NO. NAS 9-9964

Final Report

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ABSTRACT

This final report of the results of research conducted with various halocarbon compounds by the Institute of Human and Comparative Toxicology (previously, the Institute of Experimental Pathology and Toxicology) of Albany Medical College is in compliance with the requirements set forth in the contract, NAS 9-9964, and/or agreed upon between the contractor and the contract monitor.

The research reported herein constitutes primarily the conclusions reached by the contractor during this 4-year project. Detailed reports of various aspects of the work are on file with the contracting agency.

A. INTRODUCTION

Halogenated hydrocarbons are widely used in industry with the fluorinated methanes and ethanes having found important use as dielectrics, refrigerants, propellants, organic solvents, and fire extinguishers and suppressants. The compounds (termed fluorocarbons since the properties are largely governed by the fluorine moiety) are commanding an increasing market in consumer products such as food toppings, hair sprays, deodorants, etc., from their use as propellants. Although our interest in fluorocarbons was broadbased, attention was largely addressed to determining the most feasible candidate fire extinguisher and/or suppressant. When used as fire suppressants in a confined space, such as a spacecraft, aircraft, submarine, warehouse, etc., these compounds would be inhaled by man on an almost continuous basis.

In order to evaluate the safety of the candidate fluorocarbons in relation to man, we devised experiments, short-term exposures, in various animal species, to look at specific parameters in order to determine which of the candidate compounds (Table A-I) were sufficiently non-toxic to warrant further investigation, i.e., long-term exposures.

To provide the most comprehensive evaluation of the toxic-potential of each compound numerous physiologic criteria were examined: tissue distribution, fluoride ion concentration, effect on mitochondria, microsomes, liposomes and liver cell nuclei, erythrocyte fragility, clinical chemistry values, hematology, pathology, cardiac sensitization, behavioral effects, etc. It must be understood, that data for all these parameters were not evaluated for all compounds since, if a specific compound was determined to be overtly toxic, thus elminating its potential application for continuous exposure to man, further investigations generally were not conducted.

For these experiments, we used primarily rodent species for initial investigations, with non-human primate exposure for Freon 116 which was warranted by the negative results in rodents.

Species used included: rats (Sprague-Dawley), mice (Swiss albino), rabbits (Dutch Belted and New Zealand), guinea pigs, dogs, cats, and monkeys (Macaca mulatta). The choice of species was suggested by both the nature of the compound and the parameter under investigation.

Animal exposures took place in various types of chambers. The chamber selected for a particular experiment was dependent on three factors: length of exposure, species used, and compound used.

Long-term exposures were conducted in the "closed dynamic" chambers which allowed for a recirculating atmosphere and monitoring of CO_2 and O_2 levels as well as the particular halocarbon. Details of the operation are described in the Appendix (Studies in Rats Exposed Continuously to Hexachloroethane).

For short-term exposures of rodents, we used either a 100 liter metabolic exposure chamber, a 65 liter plexiglass inhalation chamber or, as in the case of the LORRT experiments, a glove-bag chamber (68 cm x 68 cm). These static chambers were sufficient for exposures which were of 30 min duration or less.

For the monkey studies, either the long-term exposure chamber or a especially-designed behavioral chamber was used. This recirculating chamber (50 cu. ft.) was designed with a behavioral-task panel and monitored by television to enable the investigator to observe the animal. The animal was unable to see out of the chamber, thus eliminating outside disturbances from effecting performance.

Table A. I. COMPOUNDS OF INTEREST

A. Substituted methanes			
	Halon 1301	Bromotrifluoromethane	$CBrF_3$
	Halon 1202	Dibromodifluoromethane	CBr_2F_2
	Freon 11	Trichlorofluoromethane	CCl_3F
	Freon 12	Dichlorodifluoromethane	CCl_2F_2
B. Substituted ethanes			
	Freon 116	Hexafluoroethane	CF_3-CF_3
	Freon 115	Chloropentafluoroethane	$CClF_2-CF_2$
	Freon 114	1, 2-Dichlorotetrafluoroethane	$CClF_2-CClF_2$
	Halon 2402	1, 2-Dibromotetrafluoroethane	$CBrF_2-CBrF_2$
	Freon 113	1, 1, 2-Trichloro-1, 2, 2-trifluoroethane	$CCl_2F-CClF_2$
	Halothane	Bromochlorotrifluoroethane	$CHBrCl-Cf_3$
C. Other			
	Freon C-318	Octafluorocyclobutane	C_4F_3

B. METABOLISM

Tissue distribution and/or blood levels of the fluorocarbon were determined following exposures of varying lengths of primarily rats and rabbits. Five fluorocarbons were used in these exposures: Halons 2402, 1201 and 1301 and Freons 113, 116 and 12. Tissues sampled included: liver, lung, heart, brain, kidney, muscle and fat. Techniques developed for analyzing the content of these fluorocarbons in the blood were developed in house. This and other analytical procedures were described herein. Results of the exposures and data accumulated are discussed by compound.

In order to determine the possibility of accumulations of F^- in bone, data was obtained from some exposures. Urine F^- concentration data are also included.

Analytical Procedures

Tissue Analysis

Due to the high volatility of the halocarbons under investigation, preliminary data of tissue concentrations revealed that recovery was only about 50%; therefore, methodologies were examined in order to determine which procedure would provide the best recovery of these compounds from organic material. The procedure outlined below was selected.

Tissue samples (1-5 g) were weighed and placed in a homogenizer. One (1) ml of 1-butanol containing 0.216 µg of Halon 2402 was added. An additional 5 ml of 1-butanol was also added and the tissues homogenized.

The homogenate was washed into a 50-ml centrifuge tube using 10 ml of isooctane, and the homogenizer rinsed with 10 ml of isooctane.

The centrifuge tube was stoppered, placed on a shaker for 30 min, and then centrifuged. A 0.5 ml sample of supernatant was removed and diluted to 50 ml using isooctane. This final solution was injected into the gas chromatograph.

A Hewlett/Packard 402 with electron capture detector was used. The 6' X 1/8' U-shaped glass column was packed with Porasil "B". The carrier gas was 95% Argon and 5% Methane. Injector port, column and detector were maintained at 260°C, 150°C and 280°C, respectively. The detector was linear between 10^{-12} and 10^{-10}g, and all readings were made in this range.

Blood Analysis

Standards. Calibration curves were prepared using static methods for making appropriate dilutions of Halon 1301 in air. Gas-tight equipment (syringes, volumetric glassware, etc.) was used throughout. The gas samples were injected into the gas chromatography with syringes. Chromatographic conditions were as follow:

Column: 6" X 3.5 mm glass packed with 80/100 mesh Porapak Q. The carrier gas was nitrogen flowing at 100 ml/min, and temperature was at 115°C. The injector and detector were maintained at 170°C and 250°C, respectively.

Recovery studies. Since gaseous halocarbons are insoluble in aqueous solutions, preparation of suitable "spiked" samples for recoveries was rendered practically impossible. To obtain some information on recoveries, experiments were carried out on samples to which known quantities of Halon 1301 were added to closed containers of blood and time allowed for diffusion of the gas into the blood. Concentration of Halon 1301 in the air above the blood was determined. The air above the blood was removed by flushing with dry nitrogen; the containers were allowed to stand for a suitable period of time,

and the concentration of Halon 1301 again determined. Using this procedure, the recovery was between 87 and 110% which we felt acceptable considering the amounts being detected and the small volumes being handled.

In all experiments using these techniques, the following precautions were taken. Vials, with rubber stopper, were weighed empty and again completely filled with water to accurately determine their volume. Actual blood volumes were computed from the weight of the sample and vial, and the density of blood.

Blood and Tissue Analyses

Halon 2402

A 30-min exposure of female albino rats (130 - 140 g) to 3.7% Halon 2402 in the metabolism chamber was followed by sacrifices of two animals at each of the indicated times. Sacrifices were at 0, 1.5, 3 and 24-hr post-exposure. Halocarbon levels in tissues were determined as previously outlined. Except for liver and blood samples taken immediately post-exposure, all samples were pooled prior to the determination. Replicate results of the liver and blood samples at 0-hour period were:

Liver: 266 and 250 μg/g

Blood: 91 and 83 μg/g

The remaining values are shown in Table B-I. In all cases except brain and fat the highest levels of the Halon 2402 were noted just after exposure, at 0 hour. In the case of the brain and fat tissue, higher levels of the agent were observed in the 1.5 hr samples indicating a shift to the lipid containing tissues. With the exception of fat, the tissue containing the highest concentration at the end of 1.5 and 3 hours is kidney which may implicate this organ in the removal of the compound from he body.

An additional 30-minute exposure with Halon 2402 (4%) was conducted using New Zealand rabbits (2 - 3 kg). The animals were sacrificed immediately following the exposure. Data from the tissues are shown in Table B-II. Highest levels of the fluorocarbon were found in the fat, which was also true in the rat experiment. Brain levels between the two species were significantly different.

Halon 1301

Four separate experiments were conducted using Halon 1301 in order to determine tissue and/or blood levels reached during the exposures. The type and length of exposure and the animal species are disccused in this section.

A 30-min exposure of two rabbits to a 5% v/v of Halon 1301 culminated in immediate sacrifice. Tissue levels for these animals are shown in Table B-III.

The remaining three studies were used to determine the blood levels of Halon 1301 after exposure. In the first, female rats (approximately 200 g) were lightly anesthetized with sodium pentobarbital and exposed to 5% v/v of Halon 1301 for 30 min. Animals were removed from the chamber and blood samples obtained by heart puncture at 0, 0.25, 0.5, 1, 2 and 4 hrs post-exposure. Blood samples were immediately transferred to closed vials and allowed to stand for 1 hour prior to analysis. Results showed a rapid decline of the fluorocarbon level in the blood. A single 50-min exposure of a rat at 5% v/v (Table B-IV) shows the decline.

The second such experiment was designed to determine blood levels of Halon 1301 following continuous exposure of 2 or 4 weeks. Female and male rats were exposed for 23 hour/day with an intermine sacrifice at 2 weeks and

final sacrifice at 4 weeks. Blood levels of the fluorocarbon were measured immediately following sacrifice. The results (Table B-IV) are consistent with the other blood-levels studies, i.e., there was no accumulation of the compound, to any significant degree, and rapid elimination of the compound resulted upon the termination of the exposure.

Therefore, in order to determine actual blood levels reached in an exposed animal, it was decided to conduct a third experiment in order to determine blood levels during actual exposure. Dutch-belted rabbits were anesthetized by intravenous administration of 40 mg/kg of nembutal and the carotid artery cannulated with polyethylene tubing. A tracheotomy was performed to prevent obstruction of the airway. The animal was placed in the 65-l bubble-shaped static exposure chamber, and the chamber sealed with the cannual leading to the outside. A baseline blood sample was drawn prior to the introduction of the Halon into the chamber. A 5% v/v of Halon 1301 was established in the chamber and fifteen samples drawn for analysis during exposure (see Table B-VI) as well as five post-exposure samples.

Halon 1202

A 30-min exposure of 4% by volume Halon 1202 was followed by immediate sacrifice of the rabbit, and tissues analyzed for the concentration of the fluorocarbon (Table B-VII).

Freon 113

A similar exposure using 4% v/v Freon 113 was conducted. Distribution of the compound in rabbit tissues is shown in Table B-VIII.

Freon 12

A single exposure of rabbit to 5% v/v Freon 12 resulted in maximum blood levels of approximately 15 μg/g. Data from this experiment are included in the Appendix (Toxicological Responses to Halogenated Hydrocarbons).

Freon 116

A single exposure during which blood levels of Freon 116 were measured is also presented in the Appendix (Toxicological Responses to Halogenated Hydrocarbons). Compared to levels seen for Freon 12 and Halon 1301 the blood levels of Freon 116 were negligible.

Fluoride Ion Concentrations

In order to evaluate the metabolic liability of fluoride in selected halocarbons, experiments were conducted to determine the F^- content of urine collected from exposed and control rats. We modified the Rowley and Farrah method (Am. Indust. Hyg. Assc. J. 23:314, 1962) to obtain greater accuracy and were still unable to obtain the precision desired; therefore, additional experiments were conducted using the Singer, Armstrong and Vogel method (J. Lab. Clin. Med. 74:354, 1969). This electrode potential measurement method with EDTA added to the diluent provided the best accuracy we were able to obtain and was subsequently used to measure the F^- concentration in urine of rats exposed to Freon 116, Freon C-318, and Halon 1301.

Regardless to whether the animals were control or exposed, the F^- content of all urine samples was very low. Examples of data obtained are shown in Tables B-IX AND B-X.

The method used for the determinations is described below.

Solutions

Standard F^-: Dissolve 2.21 g of NaF in distilled water and dilute to 1 liter. Concentration of F^- = 1000 ppm.

Working standard: Dilute 1 ml of the above standard to 1000 ml with the diluent. Concentration of F^- = 1 ppm.

Diluent: Weigh out 8.5 g NaCl and 5 g of disodium EDTA, add about 500 ml of distilled water, adjust the pH electrometrically to 6.5 with dilute KOH, and dilute to 1 liter.

Calibration of Electrometer

The electrodes used were a Coleman 3-803 (F^-) and an Orion (reference) no. 90-02-00. (The electrometer was a Sargent-Welch model NX.) The working standard (1 ppm F^-) was arbitrarily set to give a reading of -0.133 mv; when diluted 1:10 with the diluent the mv reading was -0.077. Also, when the diluent was diluted with 1/4 volume of water the mv reading was -0.025, which was equivalent to 0.008 ppm of F^- and was, therefore, negligible. The mv readings for various F^- concs. between 1 and 0.1 ppm, when plotted on semilog paper, gave a straight line. The unknowns were evaluated from this graph.

Procedure

To 1 volume of the urine sample added 4 volumes of the diluent and determined the mv reading. The routine operations were as follows:

Between samples the electrodes were washed with a stream of distilled water and dried with a sheet of tissue paper (Kimwipes). After the electrodes were immersed in the sample, an interval of 15 min (with frequent stirring) was allowed for the attainment of equilibrium. The mv reading, obtained in this way, was then converted to ppm by reference to the calibration curve, and the value so found was multiplied by 5 to give the urinary concentration (preliminary value).

Table B. I

Levels of Halon 2402 in Rat Tissue Following Exposure by Inhalation

	Post - Inhalation Interval (hr.)			
	0	1.5	3	24
Liver	258*	5	2	0.28
Lung	44	18	2	0.18
Brain	0.70	2.1	0.78	0.36
Kidney	82	27	23	0.33
Heart	24	2.1	2	1.1
Muscle	73	19	2.8	1.0
Fat	365	469	410	11
Blood	87	7	0.23	0.22

* All values shown are in µg. Halon 2402/gm tissue

Table B. II

Distribution of Halon 1301 in Rabbit Tissues*

Tissue	Tissue concentration (μg Halon 1301/g)		
	Animal #1	Animal #2	Mean
Liver	37	25	31
Lung	89	102	96
Brain	19	70	44
Kidney	21	5	13
Heart	6	21	13
Muscle	51	31	41
Fat	183	118	150

* Animals were exposed to a vapor concentration of 5% by volume for 30 minutes

Table B. III

Distribution of Halon 2402 in Rabbit Tissues*

Tissue	Tissue concentration (µg Halon 2402/g)		
	Animal #1	Animal #2	Mean
Liver	323	128	226
Lung	312	133	223
Brain	313	222	268
Kidney	101	59	80
Heart	161	121	141
Muscle	126	69	97
Fat	1048	667	858

* Animals were exposed to a vapor concentration of 4% by volume for 30 minutes

Table B. IV

Halon 1301 in Rat Blood following a single 50-minute Exposure at a 5% v/v Concentration.

Post-Exposure time (hrs.)	Blood level (μ g/g)
0	5.60
0.25	0.62
1.00	0.35
2.00	0.05
4.00	0.07

Table B. V

Concentration of Halon 1301 in rat blood following continuous exposure.

2-week exposure

Females			Males		
Animal #	Time,[a] (min.)	Conc.,[b] (µg/g)	Animal #	Time, (min.)	Conc., (µg/g)
19E	2.0	0.37	46E	4.5	0.04
25E	4.7	0.21	44E	24.0	0.01
22E	17.0	0.08	48E	31.0	0.01
23E	21.0	0.06			
control	- -	0.09	control	- -	0
control	- -	0.07	control	- -	0

4-week exposure

14E	1.5	0.12	47E	3.0	0.06
11E	3.5	0.12	50E	5.0	0.13
			34E	10.0	0.12
			41E	17.0	0.08
control	- -	0.00	control	- -	0.04

[a] elapsed time from removal of animal from exposure chamber until draining of blood sample.

[b] expressed as µg Halon 1301/g whole blood.

Table B. VI

Blood levels of Halon 1301 in a rabbit exposed to an atmospheric concentration of 5%, v/v.

	Time, min.	Conc. in blood, µg/g
	0	0.8
Start exposure		
	1.00	7.5
	2.17	4.6
	3.75	2.8
	5.00	9.7
	6.05	8.3
	7.17	7.3
	8.50	13.8
	10.33	13.1
	11.67	14.4
	13.28	15.7
	15.17	14.9
	18.00	13.9
	21.00	13.1
	25.75	9.0
	27.42	12.3
	29.67	15.3
Stop exposure		
	0.50	1.1
	1.50	0.5
	2.50	0.9
	3.83	0.7
	5.58	1.4

Table B. VII

Distribution of Halon 1202 in Rabbit Tissues*

Tissue	Tissue concentration (µg Halon 1202/g)		
	Animal #1	Animal #2	Mean
Liver	69	66	67
Lung	107	83	95
Brain	111	114	112
Kidney	70	59	64
Heart	65	63	64
Muscle	147	112	130
Fat	298	380	339

*Animals were exposed to a vapor concentration of 4% by volume for 30 minutes

Table B. VIII

Distribution of Freon 113 in Rabbit Tissues*

Tissue	Tissue concentration (µg Freon 113/g		
	Animal #1	Animal #2	Mean
Liver	27.6	27.2	27.4
Lung	21.0	10.7	15.9
Brain	26.6	20.6	23.6
Kidney	7.4	2.8	5.1
Heart	14.9	5.4	10.1
Muscle	12.3	9.8	11.0
Fat	21.0	27.2	24.1

* Animals were exposed to a vapor concentration of 4% by volume for 30 minutes

Table B. IX

Excretion of fluoride in urine and concentration of fluoride in bone of rats exposed to Freon 116 for 37 weeks

Rat no.	Water intake (ml)	Urine vol. (ml)	Urine F^- (µg/ml)	Urine F^- (µg/24 hr)	Urine F^- (µg/ml creatinine)	F^- (% bone ash)
Controls						
1	24.5	8.7	6.75	58.7	3.37	0.083
2	16.0	16.0	3.15	50.4	2.63	0.065
4	18.0	14.4	3.45	49.7	2.65	0.076
8	25.0	17.5	3.70	64.7	3.36	0.076
9	8.0	6.5	3.45	22.4	1.57	0.060
12	21.0	9.3	4.25	39.5	2.34	0.063
Mean	18.7	12.1	4.12	47.6	2.65	0.071
± S.D.	6.3	4.5	1.34	15.1	0.67	0.008
Exposed						
13	26.5	11.2	3.90	43.7	2.78	0.073
14	28.0	9.9	3.30	32.7	2.54	0.070
16	11.0	14.6	3.23	47.2	1.86	0.073
17	17.0	17.4	2.20	38.7	1.83	0.083
18	10.0	11.0	2.71	29.8	1.93	0.050
19	12.0	13.2	2.20	29.0	2.44	0.063
20	13.0	10.8	2.71	29.3	1.43	0.073
21	26.5	18.0	2.36	42.5	2.62	0.076
Mean	18.0	13.3	2.83	36.6	2.19	0.070
± S.D.	7.7	3.1	0.60	7.3	0.45	0.009

Table B. X

Concentration of free F in urine of rats continuously exposed to Halon 1301. Values as µg/ml.

1 week exposure		3 weeks exposure	
exposed	control	exposed	control
1.9	1.3	0.8	1.0
0.7	1.6	0.6	0.7
1.8	1.1	0.7	0.9
0.7	1.8	0.9	1.1
1.1	3.0	--	2.2
1.2	--	--	2.3
1.1	--	--	1.0
1.8	--	--	
1.3 ± 0.5	1.8 ± 0.7	0.8 ± 0.1	1.3 ± 0.6

C. CLINICAL CHEMISTRY AND HEMATOLOGY

Halon 1301

Forty rats (20 each male and female) were exposed in a recirculating chamber containing approximately 5% of Halon 1301 for up to 30 days (23 hr/day). Equal numbers by sex of control rats were housed outside of the chamber. Control and exposed animals received water and a commercial laboratory diet ad libitum. The Halon 1301, oxygen and CO_2 concentrations in the chamber were monitored. The average Halon 1301 concentration was 5.3%.

To determine the validity of some effects noted at the end of the 30-day exposure between control and exposed animals, a second 30-day control experiment was run. Equal numbers of controls (20 each male and female) were housed in the chamber and outside the chamber under identical circumstances except for the composition of the chamber environment. No halocarbon was used. Data from this experiment indicated that the chamber environment did, in itself, have an effect on the animals.

Comparative data was obtained from blood samples analyzed for Na^+ and K^+ content as an indicator of cell integrity and lactic dehydrogenase (LDH) (Tables C-I to C-VIII), and hemoglobin concentration, red and white cell counts, and measurement of hematocrit (Tables C-IX to C-XVI) at 1 wk, 3 wk and 4 wk. In addition, the osmotic fragility of erythrocytes was measured at 1 wk and 4 wks (Tables C-XVII to CXIX).

No effects were noted between the chamber-exposed and the chamber-control animals; although there were minor differences noted initially between the chamber-exposed and outside-control animals. Primarily, LDH values were slightly elevated in chamber-exposed animals (noted also for chamber-control animals). Secondly, mild hemoconcentration evidenced in the exposed animals was also noted in the chamber controls, but not in the outside controls. No specific sex differences were noted.

Freon 116 (Rodents)

Following preliminary exposures of rats and rabbits to Freon 116 in concentrations of up to 20% (v/v), which revealed no effect of exposure to the test species, a long-term (37 week) exposure to a 20% v/v concentration of Freon 116 was conducted in a closed dynamic chamber. Freon, oxygen and CO_2 concentrations were monitored throughout the exposure (Table C-XX). The 23-hr/day exposure allowed for cleaning and animal care.

Twelve male Sprague-Dawley rats were housed in the chamber for the exposure and an identical number housed outside the chamber as controls. The animals were weighed weekly (Table C-XX). The number of animals was reduced from the number used in the Halon 1301 exposure because of effects seen in the chamber controls which were probably due to overcrowding in the chamber. Hematology data was recorded at 18, 34 and 37 weeks of the exposure period and revealed no significant differences (Tables C-XXI to C-XXIII). Likewise, clinical chemistry data obtained at 37 weeks (which included: alkaline phosphatase, acid phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, sodium and potassium ion concentration, glucose, blood urea nitrogen and creatinine phosphokinase) are presented in Table C-XXIV and show no effect of the exposure to Freon 116.

Freon 116 (Rhesus Monkey)

Following the negative results of the 37-wk exposure in rats, a rhesus monkey was exposed to Freon 116 (20%) for 19 weeks. Chamber conditions during the exposure period are shown in Table C-XXV. Routine hematology data (Table C-XXVI) including hematocrit, hemoglobin, RBC and WBC showed no signs of change due to the exposure to the fluorocarbon. Likewise, clinical chemistry values presented in Table C-XXVII indicate no effect from the exposure.

TABLE C I

Serum biochemistry of female rats exposed to Halon 1301 for 1 week.

	Rat No.	Na+ (meq/l)	K+ (meq/l)	LDH (Units [1])
Outside Controls	1E	157	5.9	2000
	2E	153	6.9	2000
	3E	150	5.9	1880
	4E	147	5.4	1380
	5E	148	6.4	1930
	Mean	151	6.1	1840
	S.D.[2]	± 4	±0.6	±260
Exposed	16E	147	6.3	1310
	17E	149	5.8	1420
	18E	147	6.3	1410
	19E	152	6.5	2000
	20E	150	5.8	2000
	21E	153	6.0	2000
	22E	146	5.9	1910
	23E	146	5.4	2000
	24E	150	5.8	1410
	25E	150	6.6	1930
	Mean	149	6.0	1740
	S.D.	±2	±0.4	±300

[1] Berger-Boida units

[2] Standard deviation

TABLE C II

Serum biochemistry of male rats exposed to Halon 1301 for 1 week.

	Rat No.	Na+ (meq/l)	K+ (meq/l)	LDH (Units[1])
Outside Controls	26E	150	4.3	2000
	27E	149	6.0	1760
	28E	151	6.7	1490
	29E	149	6.2	1880
	30E	150	6.5	1860
	Mean	150	5.9	1800
	S.D.[2]	±1	±0.4	±190
Exposed	31E	147	4.5	1270
	32E	151	5.9	2000
	33E	146	5.8	1970
	34E	147	5.8	1220
	35E	151	5.6	1450
	36E	148	5.1	1920
	37E	155	5.8	1880
	38E	146	5.9	1420
	39E	147	5.5	1420
	40E	147	5.8	2000
	Mean	149	5.6	1655
	S.D.	±3	±0.4	±320

[1] Berger-Boida units

[2] Standard deviation

TABLE C III

Serum biochemistry of female rats confined to exposure chamber (no halocarbon) for 1 week.

	Rat No.	Na+ (meq/l)	K+ (meq/l)	LDH (Units1)	LDH_5/LDH_1
Outside Controls	1C	156	7.5	1880	2.0
	2C	155	7.1	1100	1.2
	3C	144	6.8	2820	1.6
	4C	- -	- -	1940	1.9
	5C	- -	- -	1620	1.2
	Mean	152	7.1	1870	1.6
	S.D.[2]	±7	±0.3	±620	±0.4
Chamber Confined Controls	31C	- -	- -	3400	2.0
	32C	- -	- -	2880	1.4
	33C	155	7.4	2900	1.5
	34C	160	7.4	2380	1.8
	35C	159	7.4	3180	1.5
	36C	- -	- -	1380	1.4
	37C	158	7.5	2960	1.6
	38C	- -	- -	2480	1.0
	39C	146	7.1	1580	1.4
	40C	157	7.4	1580	1.7
	Mean	156	7.4	2470	1.5
	S.D.	±5	±0.1	±720	±0.3

1 Berger-Boida units

2 Standard deviation

Table C IV

Serum biochemistry of male rats confined to exposure chamber (no halocarbon) for 1 week.

	Rat No.	Na^+ (meq/l)	K^+ (meq/l)	LDH (Units[1])	LDH_5/LDH_1
Outside Controls	41C	151	6.5	1380	1.1
	42C	154	7.3	1340	1.4
	43C	- -	- -	520	1.0
	44C	155	8.0	1400	1.3
	45C	- -	- -	1280	1.1
	Mean	153	7.3	1180	1.2
	S.D.[2]	±2	±0.8	±370	±0.2
Chamber Confined Controls	61C	- -	- -	3300	1.3
	62C	157	7.8	2040	1.7
	63C	157	7.3	2720	1.3
	64C	153	6.8	2200	1.3
	65C	151	6.7	2200	1.4
	66C	160	7.4	3480	1.4
	67C	- -	- -	3160	1.7
	68C	158	6.9	3400	1.4
	69C	- -	- -	3660	1.6
	70C	160	6.7	3680	1.3
	Means	157	7.1	2980	1.4
	S.D.	±3	±0.4	±640	±0.2

1 Berger-Boida units

2 Standard deviation

TABLE C V

Serum biochemistry of female rats exposed to Halon 1301 for 3 weeks.

	Rat No.	Na^+ (meq/l)	K^+ (meq/l)	LDH (Units[1])	LDH_5/LDH_1
Outside Controls	1E	148	6.8	1340	1.8
	2E	146	6.7	1920	1.7
	3E	147	6.6	1140	2.0
	4E	146	5.6	660	2.3
	5E	147	6.2	1680	1.9
	Mean	147	6.4	1350	1.9
	S.D.[2]	±1	±0.5	±450	±0.2
Exposed	9E	147	5.4	1960	2.0
	10E	148	5.7	2940	1.4
	11E	148	5.8	2640	1.5
	12E	150	5.7	2440	1.6
	13E	147	5.6	3540	2.0
	14E	152	5.8	1580	1.9
	15E	146	6.2	3200	1.7
	16E	148	5.6	2440	1.7
	21E	148	6.0	2940	1.2
	24E	148	5.9	1280	1.9
	Mean	148	5.8	2500	1.7
	S.D.	±2	±0.2	±720	±0.3

[1] Berger-Boida units

[2] Standard deviation

TABLE C VI

Serum biochemistry of male rats exposed to Halon 1301 for 3 weeks.

	Rat No.	Na^+ (meq/l)	K^+ (meq/l)	LDH (Units[1])	LDH_5/LDH_1
Outside Controls	26E	143	5.9	2640	1.4
	27E	146	5.9	1800	1.7
	28E	145	6.2	1500	1.5
	29E	145	6.0	2580	1.5
	30E	145	5.9	1840	1.6
	Mean	145	6.0	2070	1.5
	S.D.[2]	+1	±0.1	±510	±0.1
Exposed	31E	149	4.9	2080	2.0
	32E	152	6.5	3260	1.9
	33E	153	6.2	2440	1.6
	34E	148	5.7	3080	1.5
	35E	151	5.8	2880	1.6
	36E	150	5.1	3540	0.8
	37E	147	5.9	3080	2.0
	38E	150	5.1	3460	1.4
	39E	149	5.3	3680	1.8
	40E	147	5.7	3580	3.3
	Mean	150	5.6	3110	1.8
	S.D.	+2	±0.5	±520	±0.6

[1] Berger-Boida units

[2] Standard deviation

TABLE C VII

Serum biochemistry of female rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	Na+ (meq/l)	K+ (meq/l)	LDH (Units[1])	LDH_5/LDH_1
Outside Controls	11C	160	5.3	600	2.0
	12C	157	6.8	600	1.8
	14C	142	6.4	1100	0.5
	15C	- -	- -	860	3.2
	Mean	153	6.2	790	1.9
	S.D.[2]	±10	±0.8	±240	±1.1
Chamber Confined Controls	31C	154	6.2	2740	1.3
	34C	157	6.2	1920	3.3
	35C	160	6.9	2040	2.7
	36C	- -	- -	2640	4.4
	37C	160	6.7	2580	2.2
	38C	152	6.0	2520	2.0
	39C	160	6.6	2000	3.8
	40C	150	5.8	2580	2.1
	Mean	156	6.3	2380	2.7
	S.D.	±4	±0.4	±330	±1.0

[1] Berger-Boida units

[2] Standard deviation

TABLE C VIII

Serum biochemistry of male rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	Na^+ (meq/l)	K^+ (meq/l)	LDH (Units[1])	LDH_5/LDH_1
Outside Controls	46C	- -	- -	940	2.7
	47C	153	7.1	940	1.9
	48C	- -	- -	1000	2.5
	49C	- -	- -	810	2.8
	50C	156	7.4	380	2.2
	Mean	155	7.3	810	2.4
	S.D.	±2	±0.2	±250	±0.4
Chamber Confined Controls	71C	- -	- -	1840	2.0
	72C	158	6.4	1880	4.0
	73C	154	7.9	760	1.8
	74C	154	8.0	2040	5.0
	75C	152	6.7	2100	1.7
	76C	- -	- -	1700	3.4
	77C	157	5.8	1760	2.1
	78C	158	5.9	2100	1.8
	79C	158	6.9	2260	1.7
	80C	155	6.9	2840	1.7
	Mean	156	6.8	1920	2.5
	S.P.	±2	±0.8	±520	±1.9

[1] Berger-Boida units

[2] Standard deviation

TABLE C IX

Hematology of female rats exposed to Halon 1301 for 1 week.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Controls	1E	6.69	17.6	46.5	5.9
	2E	5.99	15.8	43.0	8.2
	3E	5.66	15.2	43.0	11.8
	4E	5.65	15.2	42.5	9.7
	5E	5.07	14.9	42.0	8.5
	Mean	5.81	15.7	43.4	8.9
	S.D.	±0.59	±1.1	±1.8	±2.2
Exposed	16E	5.88	16.0	42.5	15.1
	17E	4.96	14.7	35.0	4.5
	18E	5.07	13.6	39.0	7.8
	19E	6.21	15.8	46.5	10.4
	20E	6.40	15.7	44.0	13.1
	21E	6.39	15.7	44.0	7.0
	22E	6.00	14.9	44.0	6.1
	23E	6.07	15.5	41.5	15.1
	24E	5.43	13.6	39.0	7.8
	25E	6.02	15.0	42.5	7.5
	Mean	5.84	15.0	41.8	9.4
	S.D.	±0.52	±0.9	±3.3	±3.9

TABLE C X

Hematology of male rats exposed to Halon 1301 for 1 week.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Controls	26E	6.59	15.4	44.0	6.9
	27E	6.47	15.3	46.0	7.5
	30E	6.33	15.3	42.5	5.7
	Mean	6.46	15.3	44.2	6.7
Exposed	33E	5.95	15.4	44.0	8.7
	36E	6.51	16.2	47.0	4.8
	37E	6.06	15.2	43.0	4.2
	39E	6.12	16.5	45.0	5.9
	Mean	6.16	15.8	47.7	5.9
	S.D.	±0.24	±0.6	±1.7	±2.0

TABLE C XI

Hematology of female rats confined to exposure chamber (no halocarbon) for 1 week.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Control	1C	5.10	16.2	44.0	16.9
	3C	5.56	15.7	44.0	23.6
	4C	6.71	17.0	49.0	13.4
	Mean	5.80	16.1	45.7	17.9
Confined	32C	7.67	18.3	49.0	9.0
	34C	6.42	17.0	48.0	8.1
	35C	5.20	16.4	43.0	14.7
	36C	5.91	16.7	46.5	7.4
	37C	6.60	17.1	47.0	8.7
	38C	8.27	19.7	49.5	11.8
	39C	6.57	16.9	46.0	8.9
	40C	7.35	17.5	50.0	11.2
	Mean	6.75	17.5	47.4	10.0
	S.D.	±0.99	±1.1	±2.3	±2.4

TABLE C XII

Hematology of female rats exposed to Halon 1301 for 3 weeks.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Controls	2E	7.05	18.0	46.0	9.4
	3E	6.60	18.4	46.0	8.8
	4E	6.35	17.5	43.0	8.2
	5E	7.12	18.4	45.5	6.5
	Mean	6.78	18.1	45.1	8.2
	S.D.	±0.36	±0.4	±1.4	±1.3
Exposed	9E	7.05	17.4	46.5	12.8
	10E	7.76	17.5	49.0	14.7
	11E	5.70	16.9	44.0	10.9
	12E	7.92	18.0	48.0	11.0
	13E	8.34	18.8	50.0	14.9
	14E	7.46	17.0	47.0	11.2
	15E	6.85	17.2	46.5	5.8
	21E	7.60	17.8	47.0	12.1
	24E	8.25	17.4	47.5	10.6
	Mean	7.44	17.6	47.3	11.6
	S.D.	±0.82	±0.6	±1.7	±2.7

TABLE C XIII

Hematology of male rats exposed to Halon 1301 for 3 weeks.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Controls	26E	6.33	15.2	44.0	11.0
	27E	7.46	17.7	47.0	17.0
	28E	7.48	16.6	45.0	12.5
	29E	7.05	15.4	45.0	9.2
	30E	7.67	17.9	46.0	16.1
	Mean	7.20	16.6	45.4	13.2
	S.D.	±0.54	±1.3	±1.1	±3.3
Exposed	31E	7.33	17.4	48.0	16.4
	32E	6.63	17.7	46.5	16.2
	33E	6.47	16.4	46.0	10.3
	34E	7.43	16.2	47.0	13.9
	35E	8.63	17.3	49.0	18.5
	36E	7.15	16.8	46.5	12.1
	38E	7.77	17.4	48.0	16.9
	39E	7.02	16.6	46.0	13.1
	40E	8.46	18.7	49.0	10.5
	Mean	7.43	17.2	47.3	14.3
	S.D.	±0.74	±0.8	±1.2	±1.1

TABLE C XIV

Hematology of male rats confined to exposure chamber (no halocarbon) for 1 week.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Control	41C	6.88	16.1	48.5	11.2
	42C	5.14	15.3	42.5	21.8
	Mean	6.01	15.7	45.5	16.5
Confined	62C	5.29	15.5	44.0	9.4
	63C	7.79	16.1	46.5	12.8
	64C	7.35	16.1	47.0	16.0
	65C	6.79	18.1	50.0	8.5
	66C	7.26	16.3	46.0	10.7
	67C	6.83	17.5	51.0	12.3
	68C	7.62	16.9	47.0	6.9
	70C	8.55	17.3	48.0	15.3
	Mean	7.18	16.7	47.4	11.5
	S.D.	±0.95	±0.9	±2.2	±3.2

TABLE C XV

Hematology of female rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Control	12C	5.96	16.6	47.5	8.6
	14C	6.74	18.3	48.5	15.2
	15C	8.28	18.7	49.0	7.6
	Mean	6.99	17.9	48.3	10.5
Confined	31C	7.05	18.3	51.0	5.2
	33C	7.38	19.5	49.0	9.0
	34C	6.22	17.6	43.0	6.8
	36C	6.77	17.6	48.0	8.1
	37C	7.00	17.6	47.0	5.3
	38C	6.33	17.4	45.0	6.5
	39C	6.88	17.8	49.0	10.4
	40C	7.30	17.4	49.0	11.0
	Mean	6.87	17.9	47.6	7.8
	S.D.	±0.42	±0.7	±2.6	±2.2

TABLE C XVI

Hematology of male rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	RBC ($X10^6$)	Hb (g%)	Hct (%)	WBC ($X10^3$)
Control	46C	8.78	19.9	48.0	13.7
	47C	8.19	17.6	47.5	14.7
	48C	8.66	18.3	48.0	13.4
	49C	7.96	19.1	49.0	8.7
	50C	8.70	18.4	49.0	13.9
	Mean	8.46	18.7	48.3	12.9
	S.D.	±0.36	±0.9	±0.6	2.4
Confined	71C	7.39	18.0	51.0	11.4
	72C	7.32	17.8	48.0	9.2
	75C	7.10	16.9	46.5	5.5
	77C	7.87	18.5	52.0	7.0
	79C	6.50	16.2	44.5	6.4
	80C	6.81	16.2	48.0	6.9
	Mean	7.17	17.3	48.3	7.7
	S.D.	±0.48	±1.0	±2.8	±2.2

Table C. XVII

Osmotic fragility of erythrocytes from rats exposed to Halon 1301 for 1 week.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1E	0	1.0	46.0	97.0	99.5	100	100
	2E	0	0	7.2	74.9	97.6	100	100
	3E	0	1.0	17.8	83.9	98.0	99.5	100
	4E	0	3.8	38.8	96.2	99.4	100	100
	5E	0	0	32.0	92.2	98.8	99.8	100
	26E	0	0.5	24.1	89.4	100	100	100
	27E	0	0	19.9	85.8	99.7	100	100
	28E	0	3.0	9.0	63.8	97.0	99.8	100
	29E	0	1.0	7.0	80.2	97.8	99.9	99.9
	Mean	0	1.1	22.4	84.8	98.6	99.9	100
Exposed	19E	0	0	3.8	66.0	95.9	99.0	100
	22E	0	0	10.8	83.2	97.8	98.0	100
	23E	0	0	6.0	75.0	95.8	98.0	100
	25E	0	2.0	10.0	81.0	97.8	99.5	100
	42E	0	0	4.0	80.2	99.0	100	100
	44E	0	0	12.2	91.0	99.5	100	100
	46E	0	0	8.4	78.1	97.8	99.5	100
	48E	0	0.8	22.1	89.1	98.8	99.9	100
	Mean	0	0.4	9.7	80.5	97.8	99.2	100

Table C. XVIII

Osmotic fragility of erythrocytes from rats exposed to Halon 1301 for 1 month.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1E	0	3.0	23.5	91.0	100	100	100
	2E	0	0	18.0	84.0	99.8	100	100
	3E	0	0	23.8	95.0	100	100	100
	5E	0	0	16.8	83.0	86.8	100	100
	26E	0	0	1.5	63.6	95.2	99.1	99.5
	27E	0	0	1.0	46.0	64.2	100	100
	28E	0	0	6.0	78.0	98.0	99.6	100
	29E	0	0	0	45.9	94.1	99.5	100
	30E	0	0.2	0.6	48.0	91.2	99.0	100
	Mean	0	0.4	10.1	70.5	92.1	99.7	99.9
Exposed	11E	0	0	16.8	83.0	86.8	100	100
	14E	0	0	18.2	81.8	99.7	100	100
	15E	0	0	7.2	70.8	96.8	99.7	100
	17E	0	0	16.8	86.5	- -	100	100
	24E	0	0.2	33.1	93.4	99.1	99.8	100
	34E	0	4.8	54.2	95.2	99.2	100	100
	41E	0	0	39.0	94.0	99.8	100	100
	45E	0	8.8	63.9	96.1	100	100	100
	47E	0	0	0	57.9	95.0	99.1	100
	50E	0	0	0	72.5	96.8	99.1	100
	Mean	0	1.4	24.9	83.1	97.0	99.8	100

Table C. XIX

Osmotic fragility of erythrocytes from rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1C	0	0	9.5	99.5	100	100	100
	2C	0	0	72.8	93.8	100	100	100
	3C	0	6.4	58.2	90.0	100	100	100
	4C	0	0	46.0	85.5	99.0	100	100
	5C	0	13.7	66.2	90.8	99.3	100	100
	46C	0	0	18.2	86.8	99.0	100	100
	47C	0	0	16.0	74.6	96.2	98.2	100
	49C	0	2.8	36.6	88.8	98.2	100	100
	50C	0	0	10.1	76.8	99.5	100	100
	Mean	0	2.5	37.1	87.4	99.0	99.8	100
Confined	23C	0	0	22.7	94.4	98.5	100	100
	25C	0	0	6.8	96.3	99.2	100	100
	26C	0	0	6.0	89.4	98.6	100	100
	30C	0	0	4.0	16.8	91.0	98.5	100
	32C	0	0	33.5	85.5	97.7	100	100
	61C	0	0	11.0	87.0	98.0	99.0	100
	62C	0	0	8.6	83.6	98.0	99.5	100
	65C	0	0	25.0	88.2	96.8	100	100
	66C	0	2.2	13.5	80.6	87.1	98.8	100
	76C	0	0	5.0	65.8	94.8	100	100
	Mean	0	.2	13.6	78.8	96.0	99.6	100

TABLE C XX

Weekly Chamber Conditions and Body Weights of Rats Exposed to Freon 116

Week	Chamber conditions				Body weight, g	
	Freon, %	O_2, %	CO_2, %	T, °F	Exposed	Control
1	20.8	19.8	0.09	77	194	192
2	18.3	18.1	0.23	77	197	237
3	20.7	19.2	0.51	77	257	290
4	24.8	19.4	0.43	77	271	310
5	18.7	19.4	0.38	77	287	327
6	24.4	19.6	0.34	77	324	366
7	18.4	19.9	0.42	76	326	372
8	22.4	20.0	0.27	77	350	380
9	20.7	19.6	0.39	78	380	408
10	18.3	19.3	0.48	78	396	422
11	24.1	20.7	0.66	77	395	419
12	19.1	20.5	0.58	76	406	422
13	15.6	19.6	0.80	79	409	431
14	16.2	19.9	0.21	79	422	438
15	20.0	19.9	0.43	81	428	426
16	24.0	20.4	0.38	81	434	431
17	20.1	19.9	0.26	81	433	436
18	22.2	19.9	0.29	75	433	447
19	20.2	18.8	0.37	73	436	450
20	21.7	19.5	0.36	77	425	454
21	22.4	20.7	0.39	80	416	456
22	24.3	20.8	0.35	79	426	461
23	21.9	20.5	0.35	77	428	466
24	20.9	20.1	0.42	77	432	470
25	17.8	19.3	0.43	77	435	475
26	18.5	20.3	0.32	77	429	464
27	22.8	20.6	0.41	77	442	470
28	19.6	24.4	0.31	75	439	465
29	20.6	20.7	0.34	76	442	466
30	23.1	20.9	0.33	76	449	462
31	21.3	20.4	0.43	76	455	477
32	21.6	20.1	0.31	76	457	488
33	21.0	19.2	0.35	77	460	499
34	20.2	21.2	0.52	77	466	508
35	20.3	20.5	0.36	77	479	508
36	20.1	21.6	0.42	77	484	509
37	19.7	20.7	0.36	76	489	516

TABLE C XXI

Hematology Profiles of Rats Exposed to Freon 116 for 18 Weeks

Rat No.	Hct %	Hb g%	RBC X10^6	WBC X10^3
Control				
1	45.0	15.2	7.47	18.1
2	46.0	15.0	7.87	14.1
4	45.0	15.8	8.51	11.5
8	49.0	17.4	8.46	11.7
9	47.0	17.0	8.53	13.3
12	47.0	17.8	8.21	16.4
Mean	46.5	16.4	8.17	14.2
± S.D.	1.5	1.2	0.42	2.6
Exposed				
13	50.0	17.8	9.34	23.6
14	57.0	18.0	9.37	19.3
17	51.0	17.4	7.99	8.0
18	48.5	18.4	10.14	8.3
19	43.0	15.5	8.45	13.0
Mean	49.9	17.4	9.06	14.4
± S.D.	5.0	1.1	0.84	6.8

TABLE C XXII

Hematology Profiles of Rats Exposed to Freon 116 for 34 Weeks.

Rat No.	Hct %	Hb g%	RBC X10^6	WBC X10^3
Control				
1	50.5	19.1	8.95	7.2
2	45.0	16.8	6.77	8.0
4	48.5	18.0	8.32	7.9
8	51.0	19.3	8.16	6.0
9	48.0	18.3	8.74	6.1
12	48.5	19.3	8.51	14.2
Mean	48.6	18.5	8.24	8.2
± S.D.	2.1	1.0	0.77	3.0
Exposed				
13	47.0	18.3	7.76	8.3
14	46.0	19.1	7.19	6.1
16	50.5	20.5	8.51	7.1
17	48.0	18.3	8.03	9.7
18	50.0	18.1	8.19	9.8
19	47.5	17.6	8.05	8.3
20	51.5	19.2	9.77	10.4
21	48.5	17.6	8.63	8.3
Mean	48.6	18.6	8.27	8.5
± S.D.	1.9	1.0	.73	1.4

TABLE C XXIII

Hematology Profiles of Rats Exposed to Freon 116 for 37 weeks

Rat No.	Hct %	Hb g%	RBC x10^6	WBC$_3$ x10^3	Differential Neut	Lymph	Mono	Eosin
Control								
1	47.0	16.4	6.87	10.4	16	82	2	-
2	43.5	17.6	6.13	8.6	21	79	-	-
4	45.0	18.3	7.14	5.4	17	82	1	-
8	47.5	17.5	6.76	6.0	24	74	2	-
9	45.0	16.2	7.81	5.7	36	60	3	1
12	47.5	15.0	7.27	9.1	23	74	1	2
Exposed								
13	46.5	16.0	7.22	10.6	15	82	3	-
14	43.5	15.6	6.65	6.9	23	73	3	1
16	46.5	16.6	7.39	9.9	19	75	2	4
17	44.5	14.7	6.34	7.1	12	86	-	2
18	46.0	15.8	6.58	6.2	18	75	5	2
19	44.5	14.4	6.88	7.7	34	64	-	2
20	43.0	15.7	6.92	9.2	34	62	3	1
21	43.0	15.0	6.59	6.9	28	70	2	-

TABLE C XXIV

Chemistry Profiles of Rats Exposed to Freon 116 for 37 weeks

Rat No.	Alk.P.	Acid.P.	SGOT	SGPT	Na^+	K^+	Glu	BUN	CPK
Control									
1	13.9	9.4	84	32	144	5.2	112	18	51
2	16.9	13.5	74	20	142	5.6	120	20	76
4	6.8	12.6	62	27	146	4.9	136	14	41
8	15.8	12.0	55	17	145	5.0	140	17	25
9	9.4	9.0	55	20	145	5.1	124	18	30
12	23.6	9.2	58	50	146	5.1	156	18	6
Exposed									
13	18.6	11.4	74	20	145	5.6	120	16	20
14	35.3	11.3	70	25	145	5.2	150	14	50
16	17.8	11.4	62	11	144	5.3	128	17	25
17	26.3	11.8	90	25	144	5.6	126	14	33
18	12.8	12.0	84	30	144	5.9	126	19	53
19	26.8	9.6	58	27	145	4.8	148	13	30
20	29.6	15.6	74	27	142	5.4	168	18	38
21	24.4	5.8	21	17	143	4.7	158	20	9

TABLE C XXV

Weekly Chamber Conditions and Body Weight of Monkey Exposed to Freon 116

Week	Chamber conditions				Body weight
	Freon, %	O_2, %	CO_2, %	T, °F	kg
0	-	21	0.12	78	3.76
1	19.5	21	0.28	79	3.81
2	22.3	20	0.20	79	3.96
3	21.5	21	0.32	80	4.04
4	22.8	21	0.15	78	3.67
5	23.6	21	0.23	78	3.83
6	17.8	22	0.14	79	3.82
7	22.3	21	0.17	77	3.90
8	20.8	21	0.32	77	4.04
9	23.4	20	0.32	78	4.10
10	26.2	21	0.40	78	4.02
11	26.0	20	0.42	78	3.96
12	19.9	20	0.39	78	4.06
13	20.9	21	0.40	79	4.10
14	23.5	21	0.42	78	4.09
15	21.7	21	0.11	77	4.05
16	20.3	20	0.18	77	4.00
17	19.8	21	0.20	76	4.02
18	20.3	21	0.23	77	3.99
19	21.2	21	0.28	77	3.99

TABLE C XXVI

Hematology Profile of Rhesus Monkey Exposed to Freon 116

Date	Hct	Hb	RBC	WBC
6 Jun 72[1]	44.0	14.8	6.45	18.4
9 Sep 72[1]	45.0	14.2	6.44	22.2
13 Sep 72[1]	39.0	13.2	5.47	24.8
20 Sep 72	36.0	10.2	5.78	16.9
27 Sep 72	36.0	11.8	5.69	11.8
12 Oct 72	42.0	14 2	6.33	14.0
1 Dec 72	43.5	14.6	6.61	14.9
22 Dec 72	40.5	13.6	5.84	18.4
3 Jan 73	42.5	14.4	6.50	14.0
31 Jan 73	42.5	15.1	6.10	16.4
28 Feb 73[2]	41.5	14.6	5.40	11.5

[1]Baseline

[2]One month post-exposure

TABLE C XXVII

Chemistry Profile of Rhesus Monkey Exposed to Freon 116

Date	T.P.	ALB	CA.	I.P.	CHOL.	GLU	U.A.	CREAT.	T.BIL	ALK.P.	LDH	SGOT
6 Sep 72[1]	7.6	4.0	11.2	7.9	175	129	0.40	1.0	0.15	315	280	46
13 Sep 72[2]	7.3	3.4	10.4	6.8	148	80	0.25	0.8	0.20	312	483	49
11 Oct 72[3]	8.2	3.8	9.6	8.1	175	110	0.40	1.4	0.30	206	1880	88
22 Nov 72	7.6	3.4	10.5	5.0	162	87	0.40	0.9	0.10	203	139	29
20 Dec 72	7.5	3.9	12.0	7.9	191	68	0.20	0.9	0.10	239	285	43
3 Jan 73	7.0	3.7	9.7	3.4	146	80	0.20	0.8	0.10	197	130	30
31 Jan 73	7.0	3.7	10.1	6.2	167	90	0.30	0.9	0.20	253	289	37
28 Feb 73[4]	6.9	3.6	9.6	4.8	135	70	0.40	0.9	0.20	294	414	43

[1] Baseline

[2] Monkey in chamber 6 Sep 72 - 13 Sep 72 w/o Freon

[3] Sample badly hemolysed

[4] One month post-exposure

D. MEMBRANE SYSTEMS

In a continuing effort to assess subtle manifestations of toxicity, various studies were conducted to evaluate the in vivo or in vitro effects of halocarbon exposure on membrane systems. The specific chemicals selected for the exposures were determined by previous experimental results. They were selected for studies designed to evaluate their effect on mitochondria, microsomes, liposomes, and erythrocyte fragility. Each portion of this section discusses the development and results of these studies.

Mitochondria and Microsomes

Preliminary studies to evaluate the effect of halocarbon exposure on mitochondrial and microsomal function revealed some differences proported to be caused by the exposure; however, the methodology selected for these initial tests was thought questionable, therefore, new methods were developed (see Table D-I for a comparison of the "old" and "new" methods) and the experiments repeated.

The new method was applied to intermittent in vivo exposures involving Freon C-318 and Freon 116, short-term exposures to Halon 1301, Freon 116, Freon C-318, Freon 12 and Halon 2402, and long-term exposure to Freon 116. In vitro studies involved Freon 12 and Halon 1301.

In Vitro Studies

An experiment was designed to expose isolated mitochondria in vitro. The isolated mitochondria were placed in a respirometer with 20% v/v of halocarbon. Oxygen consumption was measured with the instrument during the actual exposure.

Studies involved exposure to Halon 1301 and Freon 12 on liver and heart mitochondria (Table D-II). No changes in mitochondrial activity were noted from these exposures.

In Vivo Studies

Short-term exposures. Female weanling rats were exposed to either Halon 1301, Freon 116, Freon C-318 or Freon 12 for 30 min or to Halon 2402 (10 min) in a static chamber. Concentrations of the halocarbons ranged from 5 - 8% v/v (see Table D-III). Control rats were placed in the chamber for an identical period but without altering normal atmosphere. Rats (control and exposed) were sacrificed immediately after removal from the chamber and mitochondria from liver, lung, brain, heart and kidney prepared and analyzed by the new procedure. Data from the analyses are presented in Table D-III. No effect on respiration or phosphorylation was indicated; however, there was a suggestion that Halon 1301 and Freon 116 and C-318 might increase the phosphorylation of brain mitochondria, or may have protected it from uncoupling during isolation. Further coverage of these data is in the Appendix (Toxicological Responses to Halogenated Hydrocarbons).

Another 30-min exposure of female rats (200 g) to Freon 12 was conducted which elicited no effect from mitochondria from heart, lung, brain or liver (Table D-IV), but indicated a possibility of a partial uncoupling of kidney mitochondria.

Long-term exposures. Following the 37-wk exposure of rats to Freon 116, various biochemical parameters of the microsomal mixed function oxidase in livers were examined. No significant effect on any of these measurements (Table D-V) was noted.

Rats exposed to Halon 1301 (5.3%) for 30 days (23 hr/day) were evaluated for effects on mitochondrial enzyme systems from heart, lung, brain, kidney and liver. Also, mitochondrial membrane activity was evaluated by measuring the respiration of NADH in the absence of any other substrate. Since NADH cannot penetrate an intact mitochondrial membrane, it was felt that this assay would be a useful indicator of the quality of the mitochondrial preparations.

The results of studies of mitochondria are shown in Tables D-VI - D-XI. No effect from exposure was indicated by any of these data. Plus, from Table D-XII, it can be seen that studies were carried out with intact, functioning mitochondria.

Microsomal enzyme systems were assessed by measuring demethylase and biphenyl hydroxylase activity. Studies were made on rat liver 12,000 x g supernatant fraction from the mitochondrial preparations. The effect of exposure to Halon 1301 on the enzyme activities are presented in Table D-XIII. Table D-XIV shows the data from identical parameters measured in confined control animals and the outside control animals. Both the exposure and/or the confinement to the chamber resulted in increases in enzyme activity; however, only in the case of 2-hydroxybiphenyl hydroxylase was the increase significant when compared to the chamber-confined controls.

Intermittent Exposures. Female rats were exposed to either Freon C-318 (5% v/v) or Freon 116 (5% v/v) for 1 hr/day, 5 days/wk for 6 wk in order to assess the effect on mitochondrial and microsomal function. Exposed and control animals were sacrificed at 2, 4 and 6 wk except for the Freon 116 exposed animals which were sacrificed at 1, 2, 4 and 6 wk.

In neither exposure was there any noted significant effect due to the exposure as can be seen from the samples of data from the Freon C-318 exposure (Tables D-XV through D-XIX). However, for this exposure, there was a suggestion

of an inhibition of oxidative phosphorylation in heart mitochondria at 4 and 6 wk. Also, for both exposures, a small consistent inhibition of liver microsomal biphenyl hydroxylase activity was noted.

Erythrocyte Fragility

To establish the effect of halocarbons on red cell erythrocyte fragility, preliminary in vitro studies were conducted, the results of which were equivocal; therefore, a second experiment using the Dacie method of measuring osmotic fragility of erythrocytes was conducted. The results of this study (Table D-XX) show a stabilizing effect on the membrane at 1 and 2 mM concentration of Halon 2402 thus confirming the earlier data.

However, these studies required a mixture of ethoxyethanol/ethanol to dissolve the halocarbon in the suspension of erythrocytes making it difficult to ascribe the observed effect to the halocarbon. Therefore, an in vivo study was conducted. Rats were exposed to 4.3% v/v Halon 2402 (10 min). The results (Table D-XXI) indicate a slight but definite increase in erythrocyte fragility.

Information was obtained during the 30-day exposure of rats to Halon 1301 on the integrity of the red cell membrane. Measurements of osmotic fragility of erythrocytes were determined at 1-wk exposure, 1-mon exposure and 1-mon confinement, i.e., chamber controls (see Table D-XXII, D-XXIII, and D-XXIV). No effect was noted from either the exposure of the animals or their confinement. These tables were also presented in Section C, as Table numbers C-XVII, C-XVIII and C-XIX.

LORRT and IT

To indirectly measure the effect of Freon 116 on microsomal function, a study was devised to measure the loss of righting reflex time (LORRT) following hexobarbital administration and induction time (IT), i.e., the time between injection and LORRT. Hexobarbital was administered to mice prior to the exposure. The effect of either a 1-hr exposure or 10-day exposure to Freon 116 was evaluated in male and female mice for the short exposure and male mice for the 10-day study.

For the 1-hr study, mice were placed in a glove bag chamber which was filled with air and Freon 116 (approximately 23%). A control group was exposed to an air/nitrogen mixture to assess the effect of decreased oxygen tension. Concentrations of oxygen, nitrogen and Freon 116 are shown in Table D-XXV.

During the 10-day exposure make-up, oxygen was replaced in the closed dynamic mode chamber and CO_2 absorbed by a LiOH scrubber. The 23-hr/day exposure allowed 1 hr for cleaning, feeding, etc. Average body weights of the animals are shown in Table D-XXVII. Table D-XXVI contains measurements of concentrations applicable to this exposure.

Following the 10-day exposure, the animals were transferred to the glove bag for LORRT and IT Measurements. Atmospheric concentrations in the glove bag were measured (Table XXV).

Although there was a variation in response to hexobarbital, the data was sufficient to demonstrate that Freon 116 had no effect on hexobarbital LORRT or IT at the exposure levels and under these conditions (Tables D-XXVIII and D-XXIX).

Liver Cell Nuclei

In order to study the effect of halocarbons on membranes, various preliminary investigations were conducted on biological membrane systems to determine the feasibility or value of extending these investigations to one or more of the halocarbons under study.

One such investigation involved the in vivo exposure of mice and rats to halothane gas followed by the homogenization of the livers upon sacrifice and subsequent isolation of a nuclear pellet for analysis for halothane (Tables D-XXX and D-XXXI). A second series of experiments using rats and mice was conducted to measure the in vivo incorporation of ^{14}C-labeled thymidine into liver DNA. Data from both these experiments are presented in Tables D-XXXII and D-XXXIII. Details of the studies were included in the First Annual Report.

Table D. I

A comparison of the 'old' and 'new methods' for assaying mitochondria

procedure	'old method'	'new method'	comment
homogenization buffer	0.25M sucrose-0.01M Tris CL	0.25M sucrose + 0.001M EGTA	EGTA removes calcium which damages mitochondria
homogenization	virtis (heart, lung) or Potter-Elvehjein (liver,kidney,brain) homogenizers	Dounce (kidney,brain heart) and Potter-Elvehjein (liver,lung) homogenizers	less shear force and less mitochondrial damage with Dounce homogenizer.
resuspension of r lets	stirring rod or homogenization	cold finger	cold finger gives homogeneous suspension with almost no shear force. Stirring rod gives poor suspension-homogenization damages mitochondria by shear force.
isolation of heart mitochondria	connective tissue broken by virtis homogenizer	connective tissue digested with an enzyme, nagarse	nagarse treatment frees mitochondria without high shear forces which damages mitochondria
assay buffer	chance media without fluoride	chance media with fluoride	fluoride inhibits ATPase whi hydrolyses ATP as it is form ed in the mitochondria. Fluoride gives higher measur ed phosphorylation.
additions to assay buffer	1.0% bovine serum albumin (BSA)	no BSA	BSA binds fatty acids and other lipophyllic compounds and prevents them from acting on the mitochondria. BSA may bind the compound whos effect on mitochondria is being measured. Hence, omission of BSA makes the mitochondria more sensitive to the compound being teste
	0.5% glucose	1.0% glucose	0.5% glucose can only trap 26% of the phosphate in the assay as glucose-6-phosphat This value is exceeded quit often in the assay. Hence, we switched to 1.0% glucose which extends the trapping capacity to 52% of the P_i.

Table D, I Cont.

(cont'd) A comparison of the 'old' and 'new methods' for assaying mitochondria

procedure	'old method'	'new method'	comment
additions to assay buffer	no glucose-6-phosphate	5mM glucose-6-phosphate	the experimental flasks trap P_i as glucose-6-phosphate while very little is formed in the blank flasks. However glucose-6-phosphatase activity is present in the mitochondria and is not corrected for in the blank flasks.
additions to sidearm	0.3 ml 0.25 M sucrose-0.01 M Tris CL to sidearm of blank flasks	0.3 ml 0.2 M KCL to sidearm of blank flasks.	KCL is better since it matches the K^+ added in the experimental flasks (they get 0.3 ml 0.1 M K_2 succinate). Chloride matches the negative charge of the succinate.
corrections	no time correction for when phosphorylation occurs but respiration is not measured	time corrected	time corrected phosphorylation gives lower but real values of phosphorylation.
P_i analysis	Fisk subbarow in the test tube	Fisk subbarow on autoanalyzer	autoanalyzer reduces human error, standardizes reagents and generally gives more reproducible results.

Table D. II

The effect of *in vitro* exposure of heart and liver mitochondria to Halon 1301 and Freon 12.

	Oxidative phosphorylation[b]	Respiration[c]	P/O[d]
Control			
Liver	1.09	0.92	1.19
Heart	2.29	1.84	1.24
Halon 1301 Trial #1[a]			
Liver	1.21	0.84	1.44
Heart	2.82	1.74	1.62
Control			
Liver	0.99	0.92	1.07
Heart	1.72	2.62	0.66
Halon 1301 Trial #2[a]			
Liver	1.10	0.86	1.28
Heart	2.42	2.31	1.05
Control			
Liver	1.39	1.35	1.02
Heart	1.34	1.70	0.79
Freon 12[a]			
Liver	1.52	1.40	1.08
Heart	1.26	1.71	0.74

a The respirometer was gassed with air or 20% Freon prior to assay.
b Expressed as μMoles P_i esterified/mg protein/20 minutes.
c Expressed as μAO/mg protein/20 minutes.
d Expressed as the ratio of oxidative phosphorylation to respiration.

Table D. III

Effect of an acute exposure to halocarbons on mitochondrial respiration and phosphorylation as measured by the improved methods

Group of Rats	Exposure Conc. %	Exposure Duration min.	Mitochondrial Activities (Succinate) Liver $O_2\downarrow$[a]	Liver $P_i \rightarrow ATP$[b]	Liver P/O[c]	Lung $O_2\downarrow$	Lung $P_i \rightarrow ATP$	Lung P/O	Brain $O_2\downarrow$	Brain $P_i \rightarrow ATP$	Brain P/O	Heart $O_2\downarrow$	Heart $P_i \rightarrow ATP$	Heart P/O	Kidney $O_2\downarrow$	Kidney $P_i \rightarrow ATP$	Kidney P/O
control	0	30	4.9	7.2	1.4	9.0	14	1.5	3.2	4.2	1.3	8.6	8.8	1.0	15	17	1.1
2402	6.2	10	6.6	8.4	1.3	12	17	1.4	2.9	3.9	1.3	10	12	1.2	15	17	1.1
control	0	30	5.8	9.5	1.7	11	18	1.8	3.0	3.7	1.2	7.7	7.5	1.0	18	21	1.2
1301	6.8	30	5.6	9.5	1.7	11	23	2.1	2.5	4.6	1.8	8.1	8.7	1.1	14	16	1.1
control	0	30	6.3	9.5	1.5	11	16	1.4	3.0	3.3	1.1	8.4	8.0	1.0	18	21	1.2
116	7.3	30	6.6	9.8	1.5	9.4	15	1.6	3.1	4.4	1.4	9.5	8.6	0.9	16	22	1.3
control	0	30	5.0	9.4	1.8	9.5	15	1.6	2.3	3.4	1.5	6.3	7.8	1.2	14	24	1.7
C-318	5.0	30	7.1	12	1.7	9.4	16	1.6	2.8	4.7	1.7	8.4	10	1.2	15	22	1.5
control	0	30	5.8	11	1.9	9.8	17	1.8	2.8	4.0	1.4	7.7	10	1.3	14	17	1.2
12	7.6	30	4.9	8.2	1.7	11	18	1.6	2.6	4.1	1.6	7.4	8.5	1.1	14	16	1.1

[a] expressed as mμAO taken up/mg protein/minute x 10^{-1}

[b] expressed as mμ Moles P_i esterified/mg protein/minute x 10^{-1}

[c] P/O= $\frac{\text{phosphorylation}}{\text{respiration}}$

Table D. IV

The effect of a 30-minute exposure to 5% (v/v) Freon 12 on mitochondrial functions.

	Oxidative phosphorylation[a]	Respiration[b]	P/O[c]	+NADH[d] / -NADH
Controls				
Heart	2.00	1.23	1.65	79/0
Lung	1.41	0.96	1.47	0/0
Brain	0.83	0.60	1.41	7/0
Exposed				
Heart	1.59	1.37	1.16	206/0
Lung	1.10	0.95	1.71	3/0
Brain	0.70	0.37	1.90	12/5

	Succinate		α-kg		β-oxy		+NADH
	ADP/O	3/4[e]	ADP/O	3/4	ADP/O	3/4	-NADH
Controls							
Liver	1.73	3.45	3.71	3.62	4.05	3.24	12/10
Kidney	1.75	2.44	-	-	-	-	10/0
Exposed							
Liver	1.72	3.36	3.92	3.04	3.24	3.04	11/11
Kidney	1.48	1.65	-	-	-	-	30/0

a Expressed as μMoles P_i esterified/mg protein/20 minutes.
b Expressed as μAO/mg protein/20 minutes.
c Expressed as the ratio of oxidative phosphorylation to respiration.
d Expressed as μAO/mg protein/min X 10^5.
e State 3 respiration over state 4 respiration.

α-kg (α-ketoglutarate) and β-oxy (β-hydroxybutyrate)

Table D. V

Biochemical parameters of the microsomal mixed function oxidase in livers of rats exposed 37 weeks to Freon 116

Biochemical parameter	Control rats (N=6)	Freon rats (N=8)
cytochrome P-450 ($O.D._{450nm}$ X 10^3/mg protein/ml)	76 ± 25	69 ± 18
cytochrome b_5 ($O.D._{428nm}$ X 10^3/mg protein/ml)	34 ± 14	35 ± 15
biphenyl hydroxylase $\left(\frac{\text{nMoles 4-OH-biphenyl}}{\text{mg/protein/hr}}\right)$	43 ± 4	36 ± 21
N-demethylase $\left(\frac{\text{nMoles p-chloroaniline}}{\text{mg/protein/hr}}\right)$	166 ± 30	146 ± 32
lipid peroxidation $\left(\frac{\text{nMoles malonaldehyde}}{\text{mg/protein/hr}}\right)$	48 ± 12	41 ± 11
glucose-6-phosphatase $\left(\frac{\text{nMoles Pi}}{\text{mg/protein/20 min}}\right)$	3.4 ± 0.9	3.8 ± 1.0

Table D. VI

The effect of continuous exposure to Halon 1301 on heart mitochondria of rats.

	Oxidative phosphorylation[a]		Respiration[b]		P/O	
	Females	Males	Females	Males	Females	Males
2 weeks exposed	1.64	1.28	0.70	1.09	2.33	1.17
controls	- -	2.16	1.69	1.91	- -	1.13
2 weeks confined	1.68	1.65	0.62	0.93	2.72	1.77
controls	1.67	1.53	0.90	0.93	1.86	1.66
4 weeks exposed	3.50	1.80	1.15	1.05	3.05	1.71
controls	4.26	2.53	1.55	1.17	2.74	2.18
4 weeks confined	2.27	1.55	1.12	0.85	2.04	1.82
controls	2.04	2.17	1.04	0.87	1.95	2.49

[a] expressed as μMoles P_i esterified/mg protein/20 minutes.

[b] expressed as μAO/mg protein/20 minutes.

Table D. VII

The effect of continuous exposure to Halon 1301 on lung mitochondria of rats.

	Oxidative phosphorylation[a]		Respiration[b]		P/O	
	Females	Males	Females	Males	Females	Males
2 weeks confined	1.57	2.47	0.89	0.92	1.77	2.69
controls	3.47	2.69	1.34	1.13	2.61	2.38
4 weeks exposed	3.12	3.36	1.07	1.26	2.93	2.66
controls	2.08	2.67	0.97	1.38	2.15	1.94
4 weeks confined	3.10	1.81	1.24	0.81	2.51	2.25
controls	2.12	1.96	0.73	0.99	3.02	1.98

[a] expressed as μ Moles P_i esterified/mg protein/20 minutes

[b] expressed as μ AO/mg protein/20 minutes

Table D. VIII

The effect of continuous exposure to Halon 1301 on brain mitochondria of rats.

	Oxidative phosphorylation[a]		Respiration[b]		P/O	
	Females	Males	Females	Males	Females	Males
2 weeks exposed	1.30	1.28	0.93	0.81	1.38	1.61
controls	1.56	0.63	0.97	0.63	1.61	1.01
2 weeks confined	0.49	0.67	0.37	0.36	1.33	1.80
controls	0.59	0.63	0.25	0.30	2.32	2.08
4 weeks exposed	- - -	1.24	- - -	0.53	- - -	2.36
controls	- - -	1.22	- - -	0.39	- - -	3.23
4 weeks confined	1.07	1.49	0.52	0.61	2.04	2.44
controls	1.36	1.95	0.43	0.61	3.15	3.71

[a] expressed as μ Moles P_i esterified/mg protein/20 minutes

[b] expressed as μ AO/mg protein/20 minutes

Table D. IX

The effect of continuous exposure to Halon 1301 on kidney mitochondria of rats.

	Oxidative phosphorylation[a]		Respiration[b]		P/O	
	Females	Males	Females	Males	Females	Males
2 weeks exposed	1.35	0.57	1.80	0.91	0.85	0.63
controls	0.96	0.57	1.70	0.92	0.57	0.62
2 weeks confined	3.90	3.26	1.72	1.87	2.27	1.74
controls	3.68	2.35	1.92	1.41	1.90	1.67
4 weeks exposed	4.01	2.22	2.45	1.80	1.64	1.23
controls	3.99	2.66	2.37	2.08	1.68	1.28
4 weeks confined	1.50	1.62	1.59	1.59	0.94	1.02
controls	2.03	1.42	1.77	1.58	1.15	0.90

[a] expressed as μ Moles P_i esterified/mg protein/20 minutes

[b] expressed as μ AP/mg protein/20 minutes

Table D. X

The effect of continuous exposure to Halon 1301 on kidney mitochondria of rats. Oxygraph measurements.

	ADP/O		3/4[a]	
	Females	Males	Females	Males
4 weeks exposed	1.44	1.58	2.52	2.10
controls	1.33	1.66	2.47	2.23
4 weeks confined	1.38	1.55	1.92	2.37
controls	1.47	1.89	2.29	2:22

[a] State 3 respiration/state 4 respiration

Table D. XI

The effect of continuous exposure to Halon 1301 on liver mitochondria of rats. Assessments with three substrates.

	succinate[a]		α-ketoglutarate		β-hydroxybutyrate	
	ADP/O	3/4	ADP/O	3/4	ADP/O	3/4
2 week exposed females	1.56	4.14	2.80	3.70	2.17	3.48
control females	1.55	4.06	2.29	3.31	2.31	4.20
2 week exposed males	1.79	3.91	2.26	4.05	2.20	3.48
control males	1.67	2.93	2.28	3.66	2.24	3.32
2 week confined females	2.14	3.80	3.16	3.04	2.26	3.42
control females	1.85	3.76	3.12	3.93	2.94	3.39
2 week confined males	1.70	3.15	2.32	2.43	2.12	2.10
control males	1.84	4.62	2.77	2.62	2.65	4.31
4 week exposed females	1.75	4.49	2.85	3.52	2.46	3.26
control females	1.65	4.39	2.70	3.37	2.59	4.17
4 week exposed males	1.98	4.28	3.38	5.05	2.98	4.81
control males	1.84	4.16	3.24	4.56	3.02	4.85
4 week confined females	1.70	3.76	2.85	2.91	2.55	2.92
control females	1.75	3.91	2.86	2.70	2.62	2.80
4 week confined males	2.08	4.16	3.34	3.09	3.28	3.74
control males	2.08	4.04	3.19	2.94	3.46	3.80

[a] state 3 respiration/state 4 respiration.

Table D. XII

The effect of Halon 1301 on mitochondrial integrity as measured by the penetrability and subsequent oxidation of NADH.

$$\frac{\text{respiration + NADH}^{a}}{\text{respiration - NADH}}$$

	Heart	Lung	Brain	Kidney	Liver
4 week exposed					
females	23/3=7.67	2/2=1	- - -	23/2=11.5	12/6=2
males	159/0=∞	- - -	5/5=1	24/1=24	10/10=1
controls					
females	29/3=9.67	5/5=1	- - -	20/3=6.67	15/15=1
males	87/5=17.4	- - -	6/6=1	14/0=∞	12/10=1.2
4 week confined					
females	69/3=23	2/2=1	7/7=1	43/5=8.6	- - -
males	79/6=13.2	5/5=1	10/10=1	7/0= ∞	12/12=1
controls					
females	74/5=14.8	3/3=1	7/7=1	36/5=7.2	- - -
males	56/6=9.33	5/5=1	7/7=1	23/7=3.28	12/12=1

[a] expressed as μAO/min/mg protein X 10^5

Table D. XIII

Microsomal enzyme activities in liver from rats exposed continuously to Halon 1301.

	exposed	controls
Demethylase[a]		
2 week females	12.25 ± 3.45	9.82 ± 2.87
males	12.07 ± 3.09	11.72 ± 2.41
4 week females	10.77 ± 1.49	7.03 ± 2.02
males	12.41 ± 1.32	10.20 ± 1.32
4-OH Biphenylhydroxylase[b]		
2 week females	0.402 ± .057	0.273 ± .023
males	0.084 ± .027	0.094 ± .011
4 week females	0.788 ± .096	0.482 ± .073
males	0.847 ± .184	0.630 ± .078
2-OH Biphenylhydroxylase		
2 week females	0.253 ± .066	0.113 ± .020
males	0.153 ± .023	0.158 ± .028
4 week females	0.387 ± .036	0.174 ± .049
males	0.379 ± .066	0.169 ± .033

[a] µg Ca/20 min/100 mg liver

[b] µ Moles/hi/g liver

Table D. XIV

Microsomal enzyme activities in liver from rats confined to the exposure chamber.

	confined	controls
Demethylase[a]		
2 week females	9.81 ± 1.04	6.90
males	12.41 ± 2.47	11.22
5 week females	6.64 ± 1.40	5.03
males	10.51 ± 1.98	11.39
4-OH Biphenylhydroxylase[b]		
2 week females	0.993 ± .129	0.654
males	0.539 ± .057	0.368
5 week females	0.245 ± .036	0.226
males	0.229 ± .047	0.260
2-OH Biphenylhydroxylase		
2 week females	0.218 ± .031	0.231
males	0.196 ± .052	0.187
5 week females	0.474 ± .084	0.392
males	0.258 ± .060	0.236

[a] µg Ca/20 min/100 mg liver

[b] µ Moles/hr/g liver

Table D. XV

Effect of Freon C-318 on heart mitochondrial respiration and oxidative phosphorylation.[1]

Weeks of exposure	Controls			Exposed		
	Oxidative phosphorylation	Respiration	P/O	Oxidative phosphorylation	Respiration	P/O
2	2.04	1.54	1.330	1.06	1.75	0.607
	1.38	1.52	0.909	2.05	1.88	1.090
4	2.22	2.46	0.899	1.20	1.90	0.631
	2.44	2.68	0.907	1.45	1.90	0.764
6	2.51	1.87	1.340	1.76	1.90	0.929
	2.09	1.88	1.120	1.58	1.91	0.828

[1] The data are expressed as follows: oxidative phosphorylation, μMoles P_i esterified/mg protein; respiration, μAO consumed/mg protein.

Table D. XVI

Effect of Freon C-318 on kidney mitochondrial respiration and oxidative phosphorylation.[1]

Weeks of exposure	Controls			Exposed		
	Oxidative phosphorylation	Respiration	P/O	Oxidative phosphorylation	Respiration	P/O
2	0.282	1.640	0.172	2.610	2.31	1.130
	0.677	1.750	0.387	2.310	2.35	0.983
4	0.598	1.000	0.595	0.706	1.23	0.574
	0.660	0.997	0.661	0.599	1.16	0.517
6	0.784	1.340	0.588	1.070	1.30	0.823
	0.892	1.330	0.670	0.945	1.25	0.755

[1] The data are expressed as follows: oxidative phosphorylation, μMoles P_i esterified/mg protein; respiration, μAO consumed/mg protein.

Table D. XVII

Effect of Freon C-318 on brain mitochondrial respiration and oxidative phosphorylation.[1]

Weeks of exposure	Controls			Exposed		
	Oxidative phosphorylation	Respiration	P/O	Oxidative phosphorylation	Respiration	P/O
2	0.687	0.570	1.20	0.530	0.482	1.10
	0.731	0.581	1.26	0.859	0.523	1.64
4	0.435	0.309	1.41	1.190	0.701	1.69
	0.395	0.307	1.29	1.190	0.714	1.66
6	0.702	0.474	1.48	0.835	0.445	1.88
	0.702	0.566	1.24	0.663	0.445	1.49

[1] The data are expressed as follows: oxidative phosphorylation, μMoles P_i esterified/mg protein; respiration, μAO consumed/mg protein.

Table D. XVIII

Effect of Freon C-318 on ADP-dependent respiration and respiratory control in liver mitochondria.[1]

Weeks of exposure	Controls						Exposed					
	Succinate		α-kg		β-oxy		Succinate		α-kg		β-oxy	
	ADP/O	3/4	ADP/O	3/4	ADP/O	3/4	ADP/O	3/4	ADP/O	3/4	ADP/O	3/4
2	1.43	3.21	2.07	2.75	1.97	2.74	1.56	3.35	2.41	3.40	2.10	3.40
4	1.44	3.25	2.19	3.01	2.03	3.26	1.50	2.88	2.26	3.27	2.16	2.70
6	1.32	2.43	2.16	2.98	2.10	3.02	1.45	2.57	2.48	3.60	2.20	3.40

[1] The data are expressed as follows: ADP/O, μMoles ADP/μAO; 3/4, state 3(electron transport limited)/state 4 (ADP limited); the substrates are succinate (not abbreviated), α-kg (α-ketoglutarate) and β-oxy (β-hydroxybutyrate).

Table D. XIX

Effect of Freon C-318 on liver microsomal biphenyl hydroxylase.[1]

Weeks of exposure	Controls	Exposed
2	0.888	0.763
	0.865	0.740
4	1.170	0.880
	1.150	0.880
6	1.180	0.988
	1.180	0.988

[1] The data are expressed as μMoles of 4-OH biphenyl produced per hour per gram of fresh liver.

Table D. XX

Dacie Osmotic Fragility Method

% Hemolysis measured as OD at 545 mu of Supematant

Salt conc. %	Control	Vehicle 12% 2-ethoxyethanol/ ethanol	Freon 2402 in 12% 2-ethoxyethanol/ethanol % Hemolysis measured as OD at 545 mu (Sup.)				
			2 mM	1 mM	0.2 mM	0.1 mM	0.02 mM
0.85	0	0	0	0	0	0	0
0.75	0.29	2.2	0.12	-0.31	0.23	0.1	-0.56
0.65	-0.48	0.67	0.23	-0.20	0.57	0.3	-0.45
0.60	0	-0.56	0.70	0.41	0.57	0.1	-0.23
0.55	0.48	0.44	0.23	0.20	1.85	0.1	-0.45
0.50	1.44	1.8	0.94	0.62	1.94	1.3	0.45
0.45	34	29	7.7	14	29	28	25
0.40	81	80	72.	70	76	76	73
0.35	85	83	73	74	79	78	76
0.30	87	96	96	90	96	95	92
0.20	100	97	102	97	94	100	97
0.10	100	100	100	100	100	100	100

Table D. XXI

*The effect of Halon 2402 inhalation (4.3% for 10 minutes) on red blood cell fragility**

NaCl concn (%)	% Hemoglobin released	
	Control rats	Exposed rats
0.85	0	0
0.75	0	0
0.65	0	0
0.60	0	0
0.55	0.8	1.1
0.50	2.7	2.7
0.48	7.2	11.7
0.40	53	65
0.35	77	89
0.30	89	93
0.20	96	98

* Determined by the method of Dacie (Miale, 1967)

Table D. XXII

Osmotic fragility of erythrocytes from rats exposed to Halon 1301 for 1 week.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1E	0	1.0	46.0	97.0	99.5	100	100
	2E	0	0	7.2	74.9	97.6	100	100
	3E	0	1.0	17.8	83.9	98.0	99.5	100
	4E	0	3.8	38.8	96.2	99.4	100	100
	5E	0	0	32.0	92.2	98.8	99.8	100
	26E	0	0.5	24.1	89.4	100	100	100
	27E	0	0	19.9	85.8	99.7	100	100
	28E	0	3.0	9.0	63.8	97.0	99.8	100
	29E	0	1.0	7.0	80.2	97.8	99.9	99.9
	Mean	0	1.1	22.4	84.8	98.6	99.9	100
Exposed	19E	0	0	3.8	66.0	95.9	99.0	100
	22E	0	0	10.8	83.2	97.8	98.0	100
	23E	0	0	6.0	75.0	95.8	98.0	100
	25E	0	2.0	10.0	81.0	97.8	99.5	100
	42E	0	0	4.0	80.2	99.0	100	100
	44E	0	0	12.2	91.0	99.5	100	100
	46E	0	0	8.4	78.1	97.8	99.5	100
	48E	0	0.8	22.1	89.1	98.8	99.9	100
	Mean	0	0.4	9.7	80.5	97.8	99.2	100

Table D. XXIII

Osmotic fragility of erythrocytes from rats exposed to Halon 1301 for 1 month.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1E	0	3.0	23.5	91.0	100	100	100
	2E	0	0	18.0	84.0	99.8	100	100
	3E	0	0	23.8	95.0	100	100	100
	5E	0	0	16.8	83.0	86.8	100	100
	26E	0	0	1.5	63.6	95.2	99.1	99.5
	27E	0	0	1.0	46.0	64.2	100	100
	28E	0	0	6.0	78.0	98.0	99.6	100
	29E	0	0	0	45.9	94.1	99.5	100
	30E	0	0.2	0.6	48.0	91.2	99.0	100
	Mean	0	0.4	10.1	70.5	92.1	99.7	99.9
Exposed	11E	0	0	16.8	83.0	86.8	100	100
	14E	0	0	18.2	81.8	99.7	100	100
	15E	0	0	7.2	70.8	96.8	99.7	100
	17E	0	0	16.8	86.5	- -	100	100
	24E	0	0.2	33.1	93.4	99.1	99.8	100
	34E	0	4.8	54.2	95.2	99.2	100	100
	41E	0	0	39.0	94.0	99.8	100	100
	45E	0	8.8	63.9	96.1	100	100	100
	47E	0	0	0	57.9	95.0	99.1	100
	50E	0	0	0	72.5	96.8	99.1	100
	Mean	0	1.4	24.9	83.1	97.0	99.8	100

Table D. XXIV

Osmotic fragility of erythrocytes from rats confined to exposure chamber (no halocarbon) for 1 month.

	Rat No.	% hemolysis in indicated salt concentration 0.55	0.50	0.45	0.40	0.35	0.30	0.20
Controls	1C	0	0	9.5	99.5	100	100	100
	2C	0	0	72.8	93.8	100	100	100
	3C	0	6.4	58.2	90.0	100	100	100
	4C	0	0	46.0	85.5	99.0	100	100
	5C	0	13.7	66.2	90.8	99.3	100	100
	46C	0	0	18.2	86.8	99.0	100	100
	47C	0	0	16.0	74.6	96.2	98.2	100
	49C	0	2.8	36.6	88.8	98.2	100	100
	50C	0	0	10.1	76.8	99.5	100	100
	Mean	0	2.5	37.1	87.4	99.0	99.8	100
Confined	23C	0	0	22.7	94.4	98.5	100	100
	25C	0	0	6.8	96.3	99.2	100	100
	26C	0	0	6.0	89.4	98.6	100	100
	30C	0	0	4.0	16.8	91.0	98.5	100
	32C	0	0	33.5	85.5	97.7	100	100
	61C	0	0	11.0	87.0	98.0	99.0	100
	62C	0	0	8.6	83.6	98.0	99.5	100
	65C	0	0	25.0	88.2	96.8	100	100
	66C	0	2.2	13.5	80.6	87.1	98.8	100
	76C	0	0	5.0	65.8	94.8	100	100
	Mean	0	.2	13.6	78.8	96.0	99.6	100

Table D. XXV

Oxygen and Freon 116 Concentrations in the Glove Bag Containing Air, Air-Nitrogen or Air-Freon Mixtures

Group	Gas Measured Concentrations in %	1 hour study		10 day study
		Males	Females	Males
Air Only	O_2[a]	19.5 ± 0.6	19.4 ± 0.9	19.2 ± 1.0
Air-N_2	O_2	16.0 ± 1.4	16.1 ± 0.9	--------
Air-Freon (116)	O_2	16.8 ± 0.3	17.2 ± 0.4	17.9 ± 1.2
Air-Freon (116)	Freon[b]	23.2 ± 2.6	22.1 ± 2.5	25.1 ± 3.6

[a] Mean ± S.D of 4 determinations (includes both trials)
[b] Mean ± S.D of 6 determinations (includes both trials)

Table D. XXVI

Oxygen and Freon 116 Concentrations in the Exposure Chambers Containing Air, or Air Freon Mixtures

Group	Gas Measured Concentration in %	Experiment 1	Experiment 2
Air Only	O_2	21.1 ± 2.3	21.8 ± 0.5
Air-Freon	O_2	21.4 ± 0.5	21.4 ± 0.5
Air-Freon	Freon	26.3 ± 3.6	22.0 ± 1.2

Table D. XXVII

Body Weights of Charles River Mice Exposed 10 days to Freon-116

	Experiment 1 Body Weight (g)		Experiment 2	
Group[a]	Initial	Final	Initial	Final
Air Only	25.7 ± 2.0	26.3 ± 1.7	25.0 ± 1.1	26.6 ± 1.3
Air-Freon 116	23.8 ± 2.7	25.4 ± 1.8	23.6 ± 2.2	27.2 ± 1.7

[a] Ten mice per group

Table D. XXVIII

Induction Time in Charles River Mice after 80 mg/kg Hexobarbital i.p.

	One Hour Study		10 day study
	Mean induction time ± S.D. (min)		
Group	Male	Female	Male
Air Only			
Exp. 1	(10)[a] 1.9 ± 0.7	(9) 2.4 ± 0.9	(10) 3.4 ± 0.9
Exp. 2	(8) 4.4 ± 2.2	(10) 2.4 ± 0.5	(10) 3.2 ± 1.2
Total	(18) 3.0 ± 2.0	(19) 2.4 ± 0.7	(20) 3.3 ± 1.0
Air-N_2[b]			
Exp. 1	(10) 2.4 ± 0.9	(10) 2.3 ± 1.1	- - -
Exp. 2	(10) 2.3 ± 0.4	(9) 2.9 ± 1.6	- - -
Total	(20) 2.3 ± 0.7	(19) 2.6 ± 1.4	- - -
Air-Freon[c]			
Exp. 1	(8) 2.3 ± 0.4	(9) 2.8 ± 1.4	(10) 2.8 ± 0.7
Exp. 2	(10) 2.4 ± 0.9	(10) 2.6 ± 1.0	(10) 2.5 ± 0.4
Total	(18) 2.3 ± 0.7	(19) 2.7 ± 1.2	(20) 2.7 ± 0.6

[a] Value in parenthesis indicates number of mice per group.

[b] See Table 1 for parameters.

[c] See Table 1 and 2 for parameters.

Table D. XXIX

LORRT in Charles River Mice after 80 mg/kg Hexobarbital i.p.

	One Hour Study		10 day study
	Mean LORRT ± S.D. (min)		
Group	Male	Female	Male
Air Only			
Exp. 1	(10)[a] 31.2 ± 12.1	(9) 26.5 ± 9.1	(10) 29.0 ± 8.7
Exp. 2	(8) 21.6 ± 8.7	(10) 20.8 ± 8.9	(10) 32.9 ±12.3
Total	(18) 26.9 ± 11.5	(19) 23.5 ± 9.2	(20) 30.9 ±10.6
Air-N_2[b]			
Exp. 1	(10) 31.5 ± 7.0	(10) 29.1 ± 8.9	- - -
Exp. 2	(10) 22.1 ± 8.4	(9) 21.9 ± 8.0	- - -
Total	(20) 26.8 ± 8.9	(19) 25.7 ± 9.1	- - -
Air-Freon[c]			
Exp. 1	(8) 21.7 ± 4.4	(9) 18.6 ± 9.6	(10) 20.2 ± 6.5
Exp. 2	(10) 22.4 ± 10.3	(10) 29.4 ± 9.8	(10) 35.2 ± 4.2
Total	(18) 22.1 ± 8.0	(19) 24.3 ± 11.0	(20) 27.7 ± 9.4

[a] Value in parenthesis indicates number of mice per group.

[b] See Table 1 for parameters.

[c] See Table 1 and 2 for parameters.

Table D. XXX

Halothane Concentrations in Isolated Mouse Liver Nuclei as a Function of Repeated Suspension and Centrifugation

Number of times separated	Halothane concentration in nuclear pellets (% of concn. in first pellet)
1	100
2	17.5
3	8.75
4*	10±0.5

* Mean of three samples

Table D. XXXI

Halothane Concentration in Liver Nuclei of Mice Exposed to 1% Halothane for One Hour

Time after end of exposure (hr)	Halothane extracted from nuclear pellet* ($g \times 10^{-11}$)	
	Freshly-exposed**	Pre-exposed**
0	5.73	12.52
1	1.64	1.05

* Nuclear pellet resuspended in sucrose (2.4 M) and separated by centrifugation.

Mean values for nuclei isolated from the livers of two mice.

** Freshly-exposed mice were untreated prior to the experiment. Pre-exposed had been exposed to 1% halothane gas 1 hr/day, 5 days/week for 16 days.

Table D. XXXII

The effects of exposure of mice to halothane vapor on relative liver weight and the incorporation of labelled thymidine into liver nuclear DNA*

Expt. no.	Halothane concn. (% by vol. in air)	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g./100g)	Specific activity (DPM/µg DNA ± S.E.)
1	0	27.0	1.54	5.70±0.14	4.14±0.55
	1.0	28.0	1.96	7.00±0.40**	3.74±0.16
2	0	27.7	1.69	6.09±0.17	lost
	1.0	24.6	1.88	7.64±0.35**	lost
3	0	28.2	1.53	5.42±0.17	5.47±1.45
	1.0	23.0	1.83	7.97±0.09**	5.96±2.07

* Halothane exposures: 1 hr per day, 5 days a week, for 2 weeks.

Four mice per group, pretreated with labelled thymidine 2 hr before sacrifice.

** Significantly different from controls ($P<0.05$ by Student's t test)

Table D. XXXIII

The effects of exposure of rats to halothane vapor on relative liver weight and incorporation of labelled thymidine into liver nuclear DNA.*

Expt. no.	Halothane concn. (% by vol. in air)	No. of rats	Pretreatment period** (hr)	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g./100 g.)	Specific activity (DPM/μg DNA ± S.E.)
1	0	4	2	151	8.29	5.49	1.02±0.24
	0	4	24	158	7.93	5.02	0.72±0.26
	0.5	4	2	166	6.34	3.82	1.26±0.54
	0.5	4	24	133	7.32	5.50	0.64±0.16
2	0	4	2	127	5.78	4.55	1.74±0.64
	0.5	4	2	121	5.93	4.90	3.28±0.76
3	0	2	2	250	13.0	5.20	4.52
	0	1 (1 lost)	24	255	13.8	5.41	8.53
	0.5	2	2	187	16.0	8.58†	1.52
	0.5	2	24	188	17.9	9.52†	1.10
	0	4	2	130	4.03	3.10	2.11±0.44
	1.0	4	2	130	4.00	3.08	1.77±0.21

* Halothane exposures: 1 hr per day, 5 days a week, for 2 weeks.
** Duration of incorporation of thymidine prior to sacrifice.
† Significantly different from controls (P 0.05 by Student's t test).

E. OTHER BIOCHEMICAL STUDIES

Synthesis and Secretion of Protein in Lung Tissue

Experiments were designed to study behavior of lung tissue at the cellular level and to ascertain its sensitivity to epinephrine following exposure to halocarbon chemicals. The study involved pulse-labeling of excised lung tissue with radioactive amino acid after the animal's exposure to a halocarbon. Secretion of protein was estimated by counting radioactive protein produced by the lung tissue. The method of Massaro, Weiss and Simon (Amer. Rev. Resp. Dis. 101:198, 1970) was used. Lung slices studies by Massaro et al., were composed primarily of cells lining alveolar sacs, and epinephrine acted in vitro to stimulate protein synthesis by these cells. Since these cells are the first to come into contact with vapors from halocarbon exposure, it was felt that the permeability of these cells and their organization might be affected by foreign materials which dissolve extensively in their membranes. To study the effect of halocarbon inhalation on the synthesis and secretion of protein in lung slices, therefore, 30-min exposures were conducted and the tissues of exposed and unexposed animals, with and without added epinephrine, were compared.

Male New Zealand rabbits (2 - 3 kg) were used for the exposure. Four animals were exposed to each of the halocarbons (Halons 2402, 1202, 1301, and Freons 113 and 116). For each exposure, one control rabbit was used.

Following the 30-min exposure, the rabbits were removed and sacrificed by stunning. Lungs were immediately removed and immersed in iced saline solution. Duplicate weighed 2 - 3 mm slices from each animal were incubated for 30 min at 37°C in 3.0 ml of oxygenated Earle's balanced salt solution containing 1 μc of uniformly ^{14}C-labeled leucine (sp. act. 251 mc/m mole). Following pulse-labeling, the slices were removed, washed with cold Waymouth

medium and placed in a vial containing 3.0 ml of oxygenated Waymouth medium. Epinephrine was added to the appropriate vials (7.0×10^{-5}M final concentration) and the vials were incubated for 15 min at 37°C. The contents of each vial was filtered and protein content of the filtrate determined by the method of Lowry, et al. (J. Biol. Chem. 193:265, 1951).

The results of protein estimation, ^{14}C counting and specific activity calculations are shown in Tables E-I through E-VI. Statistical treatment of this data in Tables E-VII - E-IX compares exposed animals with non-exposed, and epinephrine treated with un-treated.

Protein measured by the Lowry procedure showed an increase in amount secreted, but only in one case (Halon 1202) was the $P>0.05$. For those slices not treated with epinephrine (E) only Halon 2402 produced a decrease in the rate of protein secretion. For epinephrine-treated slices, Freon 113 showed a significant increase in secretion. Protein secretion measured radiometrically indicated decreases in treated and non-treated slices following Halon 2402 exposure.

Lowered specific activities reflected changes in either the rate of uptake or the rate of protein synthesis by the lung slices. The results of these experiments are shown in Tables E-X and E-XI. The effect of variation of pulse-labeling time are shown in Table E-X. The change with time of the number of counts/min/g of lung was due to ^{14}C leucine uptake and protein synthesis since secretion times were constant. The decrease observed in the chemically-measured protein could reflect either a deterioration in the protein synthesizing mechanism with time or loss of protein into the medium during labeling.

In Table E-XI, protein secretion is compared after a constant pulse-labeling period but with a varying secretion incubation time. In this case both the chemically-measured and radiometrically-measured amounts of protein

increased with time. This is also reflected in a fairly constant specific activity.

Tissue Levels of 5-Hydroxytryptamine

It was felt that measurement of tissue levels of 5-hydroxytryptamine (serotonin) might disclose a response of tissues to exposure to halocarbons. Some of the physiological responses of animals during halocarbon intoxication were strongly suggestive of central nervous system effect and serotonin is postulated to function as a neurohumoral agent. Therefore, serotonin levels were estimated in blood, brain and lung tissues of rats following exposures to various halocarbons. The procedure of Bogdanski (J. Pharmacol. Exp. Ther. 117:82, 1956) was used to make the measurements in blood, brain and lung of female Sprague-Dawley rats (100 - 150 g) after 30-min exposures to either Halon 2402, Halon 1202, Halon 1301, Freon 113, or Freon 116.

Serotonin was estimated spectrofluorometrically using a Farrand Mark I with a Farrand 7-54 primary filter and a 3-73 secondary filter. Fluorescent intensities were compared with those of appropriate standards prepared from serotonin creatinine sulfate.

Concentrations of serotonin in tissues from control animals (Table E-XII) and in tissues from exposed animals (Table E-XIII) were compared. No significant changes were observed in blood levels of serotonin. For brain levels, Halon 2402 caused an increase and Freon 113 caused a decrease. Both were significant at the 0.05 level of probability. However, the absolute value of these levels is extremely low, and it is doubtful that there was any real difference.

Serotonin levels in the lung were higher and permitted more accurate estimation. Halon 1202 caused a significant decrease in serotonin concentration while Freon 116 elicited an increase.

Table E. I

Protein synthesis and secretion in lung tissues from control rabbits

Animal #	Protein (µg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/µg protein)	
	No E*	+E*	No.E	+E	No E	+E
1	2349	1411	3624	1714	1.54	1.21
	2155	4719	1810	3360	0.84	0.71
2	1932	2989	3092	3505	1.60	1.17
	2539	3028	3740	4005	1.47	1.32
3	1974	2744	2089	2287	1.06	0.83
	2521	4669	1943	3444	0.77	0.74
4	792	3117	3311	4702	4.18	1.51
	4656	5082	4858	6463	1.04	1.27
5	2183	3277	5457	3541	2.49	1.08
	2747	3659	5137	4654	1.87	1.27
Mean	2385	3469	3506	3767	1.69	1.11
S.D.	±962	±1105	±1329	±1324	± 1.02	±0.27

* No E: without added epinephrine; +E: with added epinephrine

Table E. II

Protein synthesis and secretion in lung tissue from rabbits exposed to Halon 2402, 4% by volume for 30 minutes

Animal #	Protein (µg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/µg protein	
	No E*	+E*	No E	+E	No E	+E
1	1084	3924	680	1831	0.65	0.47
	857	1599	889	487	1.04	0.30
2	1061	2310	719	484	0.68	0.21
	1695	2963	817	1324	0.48	0.45
3	1788	3527	1853	1243	1.04	0.35
	1192	3185	1264	1139	1.06	0.36
4	738	4051	1097	1937	1.49	0.48
	346	3325	952	1564	2.75	0.47
Mean	1091	3111	1034	1250	1.15	0.31
S.D.	±478	±821	±383	±545	±0.72	±0.10

* No E: without added epinephrine; +E: with added epinephrine.

Table E. III

Protein synthesis and secretion in lung tissues from rabbits exposed to Halon 1301, 5% by volume for 30 minutes

Animal #	Protein (µg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/µg protein	
	No E*	+E*	No E	+E	No E	+E
1	2974	4429	471]	5255	1.58	1.19
	2384	3744	3290	5435	1.38	1.45
2	2853	3744	6756	10534	2.37	2.79
	1567	3209	5772	5404	3.69	1.68
3	2268	5080	2698	3570	1.19	0.70
	4953	5080	3867	3783	0.78	0.74
4	2869	5066	2111	3372	0.74	0.66
	2299	4883	1743	2607	0.76	0.53
Mean	2271	4404	3868	4982	1.56	1.22
S.D.[3]	±992	±745	±1772	±2445	±1.02	± 0.76

* No E: without added epinephrine; +E: with added epinephrine

Table E. IV

Protein synthesis and secretion in lung tissue from rabbits exposed to Halon 1202, 4% by volume for 30 minutes

Animal #	Protein (µg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/µg protein)	
	No E	+E*	No E	+E	No E	+E
1	3049	3226	2569	2488	0.84	0.77
	3230	4190	5167	2144	1.66	0.50
2	4878	4110	4615	3020	0.95	0.73
	2542	4086	4167	2227	1.64	0.67
3	2263	4522	3125	3487	1.38	0.77
	3181	4880	3300	3446	1.04	0.71
4	1471	3801	2763	4347	1.88	1.14
	3689	2764	3175	2169	0.86	0.78
Mean	3036	3948	3610	2975	1.27	0.76
S.D.	±1013	±681	±931	±761	±0.40	±0.18

* No E: without added epinephrine; +E: with added epinephrine.

Table E. V

Protein synthesis and secretion in lung tissue from rabbits exposed to Freon 116, 5% by volume for 30 minutes

Animal #	Protein (μg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/μg protein	
	No E*	+E*	No E	+E	No E	+E
1	2349	1411	3624	1714	1.54	1.21
	2155	4719	1810	3360	0.84	0.71
2	1932	2989	3092	3505	1.60	1.17
	2539	3028	3740	4005	1.47	1.32
3	1974	2744	2089	2287	1.06	0.83
	2521	4669	1943	3444	0.77	0.74
4	792	3117	3311	4702	4.18	1.51
	4656	5082	4858	6463	1.04	1.27
5	2183	3277	5457	3541	2.49	1.08
	2747	3659	5137	4654	1.87	1.27
Mean	2385	3469	3506	3767	1.69	1.11
S.D.	±962	± 1105	± 1329	± 1324	± 1.02	± 0.27

* No E: without added epinephrine; +E: with added epinephrine.

Table E. VI

Protein synthesis and secretion in lung tissue from rabbits exposed to Freon 113, 4% by volume for 30 minutes

Animal #	Protein (µg/g lung)		Activity in lung (CPM/g)		Specific activity (CPM/µg protein	
	No E*	+E*	No E	+E	No E	+E
1	1887	4483	5419	3207	2.87	0.72
	1290	4149	5226	3394	4.05	0.82
2	2395	4545	3669	3151	1.53	0.69
	2915	4851	2901	4944	0.99	1.02
3	985	6766	2500	4977	2.54	0.74
	1643	6595	2690	6365	1.64	0.97
4	3308	4323	2646	2199	0.80	0.51
	4073	5412	3399	2776	0.83	0.51
Mean	2312	5141	3556	3876	1.91	0.75
S.D.	±1062	±1025	±1160	±1402	± 1.15	± 0.19

* No E: without added epinephrine; +E: with added epinephrine.

Table E. VII

Effect of exposure of rabbits to halocarbons on protein secretion by lung tissue (as determined by the Lowry procedure).

Inhalation exposure*	Amount of protein secreted (μg/g lung tissue) No E**	+E	P value
Control	2385±962	3469±1105	<.05
4% Halon 2402	1091±478 P <.01	3111±821 P <.5	<.01
5% Halon 1301	2771±992 P <.5	4404±745 P <.4	<.01
4% Halon 1202	3036±1013 P <.1	3948±681 P <.1	<.1
5% Freon 116	3010±575 P <.2	4072±1109 P <.4	<.05
4% Freon 113	2312±1062 P <.9	5141±1025 P <.01	<.01

* All exposures were for 30 minutes

** No E: without added epinephrine; +E: with added epinephrine.

Table E. VIII

Effect of exposure of rabbits to halocarbons on protein secretion by lung tissue as determined by the radiometric procedure.

Inhalation exposure*	Activity in lung (CPM/g) No E**	Activity in lung (CPM/g) +E	P value
Control	3506±1329	3767±1324	<.07
4% Halon 2402	1034±383 P <.01	1250±545 P <.01	<.4
5% Halon 1301	3868±1772 P <.7	4982±2445 P <.2	<.4
4% Halon 1202	3610±931 P <.9	2975±761 P <.2	<.2
5% Freon 116	4396±1470 P <.2	4634±1204 P <.2	<.8
4% Freon 113	3556±1160 P <.9	3876±1402 P <.9	<.7

* All exposures were for 30 minutes

** No E: without added epinephrine; +E: with added epinephrine.

Table E. IX

Effect of exposure of rabbits to halocarbon on the specific activity of protein secreted by lung tissue

Inhalation exposure*	Specific activity of secreted protein (CPM/μg protein)		P value
	No E**	+E	
Control	1.69±1.02	1.11±0.27	<.1
4% Halon 2402	1.15±0.72 P <.2	0.31±0.10 P <.01	<.01
5% Halon 1301	1.56±1.02 P <.8	1.22±0.76 P = .4	<.4
4% Halon 1202	1.27±0.40 P <.3	0.76±0.18 P <.01	<.01
5% Freon 116	1.51±0.54 P <.6	1.17±0.28 P <.2	<.1
4% Freon 113	1.91±1.15 P = .6	0.75±0.19 P <.01	<.02

* All exposures were for 30 minutes

** No E: without added epinephrine; +E: with added epinephrine.

Table E. X

Effect of pulse-labeling time on the amount of protein secreted in lung slices from rabbits treated with Halon 2402.

Treatment	Pulse labeling time (min.)	Amount of secreted protein		Sp. act. (CPM/µg protein)
		µg protein/g lung *	CPM/g lung	
Control	15	2222	1644	0.74
	30	1355	3577	2.64
	60	546	8974	16.44
	120	889	9707	10.92
Control + added epinephrine	15	3250	2740	0.84
	30	2929	5071	1.73
	60	2972	9401	3.16
	120	3374	18630	5.52
4% Halon 2402 (30 min)	15	1774	1139	0.64
	30	1852	2403	1.30
	60	499	6344	12.71
	120	122	7220	59.18
4% Halon 2402 + added epinephrine	15	2675	1160	0.43
	30	1884	2030	1.08
	60	3846	4757	1.24
	120	4524	12800	2.83

* Lowry procedure

Table E. XI

Amount of protein secreted as influenced by period of active protein secretion in lung slices from rabbits treated with Halon 2402.

Treatment	Time (min.)	Amount of secreted protein		Sp. act. (CPM/µg protein)
		µg protein/g lung*	CPM/µg lung	
Control	15	2513	1918	0.76
	30	3630	2260	0.62
	60	3941	3313	0.84
	120	8396	8066	0.96
Control + added epinephrine	15	2581	2145	0.83
	30	4218	1790	0.42
	60	5836	3568	0.61
	120	-------Sample Lost------		
4% Halon 2402 (30 min.)	15	2075	830	0.40
	30	1756	719	0.41
	60	3090	1957	0.63
	120	6645	2558	0.38
4% Halon 2402 + added epinephrine	15	4158	917	0.22
	30	3374	1125	0.33
	60	4298	1474	0.34
	120	6538	2735	0.42

* Lowry procedure

Table E. XII

Serotonin (5-hydroxytryptamine) levels in control rat tissues

Serotonin levels (µg/g)		
Blood	Brain	Lung
1.5	0.8	(10.2)
0.7	0.8	3.9
1.7	0.8	2.4
0.9	0.5	2.9
1.1	0.6	4.9
1.1	0.5	1.5
0.4	0.5	4.4
1.0	0.7	3.4
1.4	0.4	3.9
	0.6	4.1
	0.5	4.4
	0.5	4.3
	0.6	4.6
	0.5	1.4
	0.5	4.2
		4.3
		3.1
1.1±0.4*	0.6±0.1	4.0±1.2

* Mean ± standard deviation

Table E. XIII

Serotonin (5-hydroxytryptamine) levels in rats exposed to various halocarbons for 30 minutes

Halocarbon	Concn. in air (by vol.)	Serotonin levels (μ g/g) Blood	Brain	Lung
		1.1	0.8	2.1
		0.6	0.7	(10.2)
Halon 2402	4.0%	0.8	0.7	2.9
		1.1	0.7	4.7
		1.0	0.7	4.7
	Mean ± S.D.	0.9±0.2	0.7±0.1	3.8±3.1
		1.1	0.5	1.0
		1.1	0.5	3.7
Halon 1202	2.0%	0.5	0.6	1.1
		1.1	0.5	3.3
		1.0	0.5	1.1
	Mean ± S.D.	1.0±0.3	0.5±0.1	2.0±1.3
		0.6	0.6	3.3
		1.1	0.5	5.3
Halon 1301	5.0%	1.0	0.5	4.9
		0.7	0.5	4.2
		0.6	0.6	2.7
	Mean ± S.D.	0.8±0.2	0.5±0.1	4.1±1.2
		0.8	0.5	4.3
		0.6	0.4	1.5
Freon 113	4.0%	1.3	0.5	7.8
		0.5	0.4	3.9
		--	0.4	2.6
	Mean ± S.D.	0.8±0.4	0.4±0.1	5.7±2.4
		0.4	0.5	8.6
		0.8	0.5	5.8
Freon 116	5.0%	0.5	0.4	9.3
		0.9	0.6	6.6
		1.0	0.5	6.8
	Mean ± S.D.	0.7±0.3	0.5±0.1	7.4±1.5

F. BEHAVIORAL EFFECTS

During the first year of study, behavioral changes were noted during 30-min exposures of rats and/or rabbits to Halon 2402, Halon 1202 and Freon 113 at levels of 3-to-4% by volume. However, 30-min exposures of rabbits to Halon 1301 and Freon 116 elicited no such response.

Since results of clinical observations, as well as many physiologic parameters were predominantly negative for Freon 116 and Halon 1301, they were selected for use in a behavioral task using a mature male rhesus monkey. This study, incorporating an instrumental task, was employed in an effort to detect subtle behavioral changes as a function of the fluorocarbon concentration. A chamber was designed to hold a single animal during the exposure with monitoring both by computer, which programmed the task and recorded each response, and by television to detect gross behavioral changes. No gross differences were noted; however, response data obtained suggested that for these two compounds, a differential effect upon certain behavioral components at reasonably high levels of concentration (35-to-50%) did occur. A general discussion of this study was included in an earlier report. Although the results were interesting, we are aware that more extensive testing would be necessary to corroborate these findings.

G. HISTOPATHOLOGIC FINDINGS

Single, Acute Exposures

Four groups of six female rats were sacrificed immediately after 30-min exposures to either Freon 116 (5% v/v), Freon C-318 (5% v/v), Halon 1301 (5% v/v) or Halon 2402 (4% v/v). The animals were killed by servical dislocation. All three body cavities were opened and the contents examined in situ. Heart, brain, lungs, liver, kidney and spleen were removed and fixed in buffered formaldehyde solution. Tissue samples were embedded in paraffin and stained with hematoxylin-eosin. Some frozen sections of liver were stained with an oil red 0 method. The results are summarized as follows:

Respiratory system. There were no gross or microscopic changes in the trachea, bronchial tree and pulmonary parenchyma that could be attributed to inhalation exposure.

Central Nervous System. A slight, foamy vacuolisation was found in the ground substance of the frontal area of the brain, indicating a slight degree of edema. The incidence was 4/6 for all compounds except Halon 1301. Samples were not taken at autopsy from the Halon 1301 animals.

Heart. In one rat (Freon 116) some vacuolisation of the mycardial fibers was seen. The cardiac muscle from all other animals were normal.

Liver. No gross abnormalities were seen. Microscopically some degree of fatty infiltration was seen.

Spleen and kidney. No gross or microscopic changes were noted in any animal.

Freon 116 Multiple Exposures

Twenty female rats were exposed to a concentration of 5% Freon 116 1 hr daiiy for 9 days. All animals were killed by cervical dislocation 48 hrs after the last exposure. Complete autopsies were performed and samples of heart,

trachea, lung, esopahgus, stomach, small and large intestines, liver, pancreas, kidney, urinary bladder, ovary, uterus, pituitary, thyroid, adrenal, spleen, thymus, bone marrow, cerebrum, cerebellum, eye and skeletal muscle were fixed in neutral buffered formaldehyde. Paraffin sections 6μ thick, were stained with hematoxylin-eosin, and examined microscopically.

All organs examined except brain, liver and lung were grossly and microscopically normal. For the lung, 10 of the 20 animals examined had slight edema surrounding middle-sized branches of the pulmonary veins. For the cerebrum, 12 rats showed a slight edema of the frontal lobe. For the liver, 18 rats showed a slight vacuolation of the liver cells in the portal area.

Freon 116 Long-term Exposure

Following the 37-wk exposure of rats to Freon 116 (20% v/v), eight exposed males and six control males were sacrificed and five organs (heart, lung, kidney, liver and brain) processed and examined microscopically.

All rats were sacrificed (ether inhalation) and immediately after death all body cavities were opened and the contents examined in situ. Representative samples were fixed as previously described, and stained with H&E.

All livers, hearts and central nervous system sections were grossly and microscopically normal. Various minimal to slight degrees of chronic murine pneumonia were found in almost all exposed and control animals. One case of focal vascular calcification was found in an exposed animal. For the kidneys, one case of moderate interstitial nephritis (control animal) was noted. Two exposed rats had a few optically empty cysts in the cortex and cortico-medullary border. An additional exposed rat had bilateral hydronephroses with thinning of the cortex and medulla.

All morphological changes in the lungs and kidneys are known as spontaneous diseases in rats and could not be attributed to the exposure to Freon 116.

H. CARDIAC SENSITIZATION

Various studies were conducted in order to determine the effect of halocarbon inhalation on the cardiovascular system. Guinea pigs, cats and dogs were used throughout the experiments to determine the ability of the compounds to sensitize the heart to infused or injected epinephrine.

The six halocarbons selected for study were two Halons (1301 and 2402) and four Freons (11, 12, 116, and C-318). Results of the in vivo work are shown in the Appendix (Sensitization of the Heart to Catecholamine-Induced Arrhythmia and Studies of Sensitization of Cardiac Muscle to Epinephrine by Selected Halocarbon Compound).

In addition to the in vivo exposures, an in vitro study using Freon 11 was conducted and the results presented in the Appendix (The Effects of Freon 11 and Certain Drugs on Isolated Auricles).

APPENDICES

1. TOXICOLOGICAL RESPONSES TO HALOGENATED HYDROCARBONS

   Travis Griffin and James Byard

2. USE OF ANIMALS IN EXPERIMENTS TO PREDICT HUMAN RESPONSE

   Travis B. Griffin and F. Coulston

3. STUDIES IN RATS EXPOSED CONTINUOUSLY TO HEXAFLUOROETHANE

   Travis B. Griffin, J.L. Byard and F. Coulston

4. STUDIES OF SENSITIZATION OF CARDIAC MUSCLE TO EPINEPHRINE BY SELECTED HALOCARBON COMPOUNDS

   J. Henry Wills

5. SENSITIZATION OF THE HEART TO CATECHOLAMINE-INDUCED ARRHYTHMIA

   J. Henry Wills

6. THE EFFECTS OF FREON 11 AND CERTAIN DRUGS ON ISOLATED AURICLES

   J. Henry Wills
   D. Sibler

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TOXICOLOGICAL RESPONSES TO HALOGENATED HYDROCARBONS

Travis Griffin and James Byard

Paper Presented at a Symposium on
"An Appraisal of Halogenated Fire Extinguishing Agents"
NAS/NRS Washington, D.C.
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Halogenated hydrocarbons are widely used in industry with the fluorinated methanes and ethanes having found important uses as dielectrics, refrigerants, propellants, organic solvents and fire extinguishers and suppressants. These compounds (termed fluorocarbons since the properties are largely governed by the fluorine moiety) are commanding an increasing market in consumer products such as food toppings, hair sprays, deodorants, etc., from their use as propellants. Although our interest in fluorocarbons is broadbased, our attention has largely been addressed to the study of candidate fire extinguishers and fire suppressants. The list of compounds we have investigated incorporates 11 different halogenated hydrocarbons, including the anesthetic halothane (see Table 1), but most of our attention has been directed to studies of bromotrifluoromethane (Halon 1301) and hexafluoroethane (Freon 116). Some detailed attention has also been given to 1,2-dibromotetrafluoroethane (Halon 2402), dichlorodifluoroethane (Freon 12) and octafluorocyclobutane (Freon C-318).

Exposure of animals by the route of inhalation is important since the compounds are either gasses or highly volatile liquids at ambient temperatures and pressure. This route of administration was chosen for the majority of our studies. Our investigations have involved the use of three different types of inhalation exposure in laboratory animals. In the first, animals have been exposed for a single short period of time (a few minutes up to one hour) and the immediate effects of exposure assessed. Exposures of this type have been widely used in studies of effects on biochemical systems, pharmacologic effects and studies of metabolism and tissue distribution of the compounds. The results we have obtained in the single short-term exposures will be emphasized in this presentation. Other

investigations have employed the use of multiple intermittent exposures and we are currently conducting studies in which animals were exposed continuously for periods up to 23 hours per day or longer in environmentally-controlled exposure chambers.

In deciding on the type of exposure to be employed in a given experiment we considered how humans would be exposed as a result of the intended use of the halocarbon. Bromine-containing fluorocarbons, such as Halon 1301, are good fire extinguishers and have been studied from the standpoint that they will be used in situations resulting in brief exposures of humans. They have not received extensive study in continuous exposure situations. However, if such a compound were used in an enclosed atmosphere such as that of a spacecraft, it could remain as part of the atmosphere for an extended period and occupants of the vehicle would be in a continuous exposure situation. On the other hand, compounds such as Freon 116, which have been proposed for use as fire suppressants, might deliberately be incorporated into the spacecraft atmosphere resulting in continuous exposure of human beings.

With regard to immediate toxic effects as judged by behavioral responses of laboratory animals during exposure, our studies have shown that animals appeared to tolerate well single as well as multiple exposures to Halon 1301, Freon 116 and Freon C-318. Halon 1301 and Freon 116 are both well-tolerated by rats and guinea pigs at atmospheric concentration as high as 20%. We have not studied higher concentrations of these compounds in intact, unanesthetised animals. By contrast, laboratory animals are less able to tolerate exposures to 5% concentrations of Halon 1202, Halon 2402, or Freon 113. All three of these compounds caused convulsive movement in rabbits, and Halon 1202 caused violent convulsions and death in rats.

These observations are in accordance with the generally-accepted principle relating the chemical constitution with the toxicity of many of the fluoroalkanes, namely a lower toxicity is associated with an increasing number of fluorine atoms in the molecule (Clayton, 1966). The low order of toxicity in highly fluorinated alkanes is a reflection of low-chemical reactivity and low-biological activity. Since one of the goals of our research is to select at least one compound suitable for controlled, continuous exposure in human volunteers, our more detailed investigations have centered largely around less toxic compounds.

One biological action of halocarbons which is well recognized is the phenomenon of sensitization of the myocardium to epinephrine. Studies of these effects are extremely important and are covered in greater detail elsewhere in this symposium. Suffice it to say that we are including studies of cardiac sensitization in our general investigations on fluorocarbon toxicity and that our results are consistent with the findings of others in this area. Compounds containing chlorine such as the Freons 11 and 12, are among the most potent sensitizers investigated while the perfluorinated compounds, for example Freon 116, are among the least active cardiac sensitizers.

Because of the non-polar character and subsequent high degree of lipid solubility of the compounds under investigation, it is conceivable that they may exert effects on biological lipid membrane systems. In an investigation of this possibility, we directed some of our studies to the effects on mitochondrial function in tissues obtained from animals exposed to fluorocarbons. Some of our early investigations suggested that exposure of rats to halogenated hydrocarbons may have some effect on respiration and oxidative phosphorylation in isolated mitochondria. However, more thorough investigations

failed to substantiate any such effects. The results of studies of effects on oxygen consumption are shown in Figure 1. In these studies, mitochondrial functions were measured in animals which were sacrificed immediately following a single exposure to the halocarbon under the conditions shown in the Figure. It can be seen that, in general, the levels of oxygen consumption in mitochondria from the exposed animals were within the range of control values obtained for each of the five tissues studied. A similar pattern of response was seen for oxidative phosphorylation shown in Figure 2. There is, however, a suggestion of enhanced mitochondrial function, e.g., phosphorylation in mitochondria from brain of animals exposed to Halon 1301, Freon 116 or Freon C-318. The effect, if real, more likely means that the compounds may have protected the mitochondria from uncoupling during isolation. Further investigation would be required to substantiate this observation.

Preparation of mitochondria for assay of enzyme activities requires homogenization and centrifugation techniques. These treatments certainly reduce, if not entirely eliminate, the content of gaseous halocarbons in these preparations. In an attempt to overcome these difficulties, an experiment was designed to expose isolated mitochondria *in vitro* to the halocarbons. In order to accomplish this, the isolated mitochondria were placed in the respirometer, but the air in the respirometer was replaced with air containing 20% by volume of either Freon 12 or Halon 1301. Oxygen consumption was then measured with the instrument as usual, but during the actual exposure to the halocarbons. Studies carried out with liver and heart mitochondria did not demonstrate any changes in oxidation or phosphorylation.

We have also examined mitochondrial function in animals exposed continuously to fluorocarbons. In an experiment involving exposure of rats

continuously to an average of 5.3% Halon 1301 for one month (*vide infra*) we also failed to demonstrate any decrement in mitochondrial function in heart, lung, brain, kidney or liver.

Although our efforts to date have not revealed strong interactions between halocarbons and oxidative enzyme systems of mitochondria, work by others has demonstrated the formation of a cytochrome P-450 substrate complex with perfluoro-n-hexane (Ullrich and Diehl, 1971). The system used was a rat liver microsomal preparation. It was shown that perfluoro-n-hexane in contrast to n-hexane was not hydroxylated by the microsomal monooxygenase system but that the fluorocarbon formed an enzyme-substrate complex and stimulated NADPH oxidation. Hydrogen peroxide was not formed and it was concluded that the fluorocarbon acted as a typical dead-end inhibitor of the microsomal monooxygenase system and lead to the uncoupling of electron transport from monooxygenation.

Any biological response to a fluorocarbon, whether it be sensitization of the myocardium or any other manifestation of effect, requires transport of the agent to the primary site of action. Assessment of this can be made through measurement of tissue levels. In our first such experiments, attempts were made to measure concentrations of the halocarbons in the tissues of animals immediately following exposure to the compounds. The results of such a study in which rats were exposed to Halon 2402 are shown in Table 2. In this experiment, the rats were exposed to 3.7% vapor concentration of Halon 2402 for 30 minutes. Immediately following the exposure, and at intervals thereafter, pairs of rats were sacrificed and tissue levels of Halon 2402 measured. Detectable concentrations of the compound were present in the animal even 24 hours after exposure. The increase of concentration of Halon 2402 in brain tissue and fat tissue

demonstrates that the 30-minute exposure to the compound was not long enough to establish an equilibrium in the animals. The half-life of the compound in blood was less than one hour.

One interesting aspect of this study concerned the relationship, or lack thereof, between levels of Halon 2402 in the brain and the CNS effects as evidenced by behavior. Although behavior patterns returned to normal within 15 minutes after cessation of exposure, brain levels were apparently higher 1.5 hours after exposure and were significantly elevated even 3 hours after exposure.

Since blood is a readily accessible tissue, and may be obtained without sacrificing the animal, additional studies were made in blood to compare the absolute amount of halocarbon following exposure and the half-life in blood. The results of such a study with Halon 1301 are shown in Table 3. In this investigation, anesthetized rats were placed in the chamber and exposed to a vapor concentration of 5% Halon 1301 for 30 minutes. Levels of Halon 1301 were then determined in blood which had been obtained by heart puncture. These data show that the half-life of Halon 1301 is much less than the 30-minutes observed for Halon 2402. Accurate assessment of half-life from these data is not possible since the 0-time was actually a period 0.5 minutes to 1.5 minutes following removal of the animals from exposure since this amount of time was required to obtain the blood samples.

It should be noted at this time that in order to obtain any meaningful results at all with a gaseous halocarbon, it was necessary to utilize methods representing a significant departure from our usual procedure. Whereas with the liquids, such as Halon 2402, solvent extraction techniques provided quantitative results, severe losses were observed when the techniques were applied to the gaseous halocarbons. In order to obtain

quantitative data with the gaseous compounds, it was necessary to employ techniques involving head-space analysis. In these procedures, blood was withdrawn from the animals by techniques which did not allow the blood samples to come in contact with air. The sample was then transferred quantitatively to a small vial which was sealed with a septum. After a suitable period to allow equilibrium conditions to be obtained within the vial, the gaseous halocarbon was quantitatively determined in the air space within the vial itself.

Previously we had withdrawn animals from exposure chamber, sacrificed them, and removed blood for analysis. In our studies with Halon 1301, using the improved head-space analysis technique, it soon became evident that more suitable approaches would be required in making meaningful estimations of blood levels of low boiling materials during the exposure of the animals. The data indicated that very rapid decreases in blood concentrations occurred during the first few minutes after cessation of exposure. The time required to obtain the blood sample after the animal was removed from the chamber was long enough, in relation to the half-life of the gasses in blood, to preclude accurate estimation of blood levels during exposure. In order to overcome these difficulties, a different type of procedure was followed.

For these studies, Dutch-belted rabbits were chosen as the experimental animal since they were large enough to allow a sufficient number of blood samples to be obtained yet small enough for the static exposure chamber employed. The animals were anesthetized by means of intravenous administration of nembutal and the carotid artery was cannulated with polyethylene tubing. A tracheotomy was performed to prevent any possible obstruction of air way. The animal was placed in the exposure chamber and the chamber sealed with the arterial cannula leading to the

exterior. A blood sample was then withdrawn to establish the baseline condition. Halocarbon vapor was then admitted to the chamber in the desired concentration and blood samples were collected periodically during the exposure. Finally, the chamber was opened, effectively lowering the fluorocarbon concentration to zero, and blood samples were taken for several minutes thereafter. Halocarbon concentrations were then determined in the blood samples by the head-space analysis technique previously described. The results of two experiments, one with Halon 1301 and the other with Freon 116, are shown in Figure 3. The study with Halon 1301 showed that blood levels as high as about 15 μg/g were encountered during exposure to 5% atmospheric concentrations of the halocarbon. Our previous work had indicated blood levels of only about 5 μg/g immediately after exposure. The reason for this difference is apparent when one considers the rapidity with which blood levels followed changes in exposure conditions. Within 30 seconds of the time the animals were removed from the chamber, the blood level dropped from 15 μg/g to insignificant levels. This demonstrates the importance of obtaining a blood sample during the actual exposure to the halocarbon and not a few moments afterwards. Events during the start of the exposure were not so straightforward. Stabilized blood levels were not reached until about ten minutes after the start of the exposure. While it is conceivable that this period was required to establish an equilibrium condition within the animal, a more likely explanation is that the time was required to establish an equilibrium within the exposure chamber. Indeed, analysis of the air within the chamber at various points indicated that stable conditions were not achieved until after about 15 minutes. Contrastingly, at the end of the experiment, the animal was removed from the chamber, and admitted instantaneously to a stable (0 halocarbon concentration) atmosphere.

Data obtained during a similar exposure to Freon 116 at 5% showed less fluctuation during the initial stages of the exposure but more importantly demonstrated that the highest levels of this halocarbon reached in the blood of the animals during exposure was less than 1 μg/g. In fact, during exposure, the concentration ranged from 0.2 to 0.6 μg/g. As in the case of Halon 1301, blood levels of Freon 116 decreased to unmeasurable levels within one or two minutes after the animals were removed from the exposure chamber. We have also demonstrated a rapid equilibration and short half-life in rabbit blood with the gaseous fluorocarbon Freon 12. The results of a study with this compound are shown in Figure 4. In this study a circulating fan was incorporated into the chamber to establish as rapidly as possible a stable exposure condition. A short half-life in blood also has been demonstrated for Freon 11 (Patterson, Sudlow and Walker, 1971). A half-life of 0.5 to 1.5 minutes was measured in humans using pressurized aerosols of adrenergic bronchodilator drugs.

Our laboratory is now undertaking studies of continuous exposure of laboratory animals to selected fluorocarbons. A 30-day exposure of rats to 5% Halon 1301 has recently been completed. In the study, the animals were exposed continuously for 23 hours each day in an environmentally-controlled exposure chamber. The remaining time each day was used for maintenance and animal care within the chamber. Extensive studies failed to reveal effects on serum electrolytes or hemotologic parameters including erythrocyte fragility in the exposed animals. As indicated previously, mitochondrial function was not altered.

Studies of blood did not reveal any accumulation of Halon 1301, as the material was apparently cleared from the continuously-exposed animals as rapidly as it was cleared from animals exposed only for a few minutes.

There was no elevation in the rate of excretion of fluoride ion in the urine. There was some indication of elevated levels of liver enzymes although the results were not conclusive. Grossly, there was no pathology at autopsy and a thorough histological examination is underway. We are currently conducting a study in which rats are being exposed in a similar manner to 20% Freon 116.

To summarize, we have investigated a number of conceivable biological effects of selected halogenated hydrocarbons in laboratory animals exposed briefly to the compounds. Similar studies are underway in continuously-exposed animals. With the exception of cardiac sensitization and within the limits of our exposure conditions, only minimal effects have been observed with the highly fluorinated compounds (Freon 116 and Halon 1301). While these observations support the general concept of biological inactivity of highly fluorinated compounds, we still hesitate to apply the term "biologically inert". We plan studies employing procedures which, hopefully, will provide more sensitive probes into the biological action of fluorocarbons.

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TBG:r

REFERENCES

Clayton, J. W., *Handbuch der experimentellen Pharmakologie*, Vol. XX (O. Eichler, A. Farah, H. Herken, and A. D. Welch, eds.) Springer, Berlin, 1966, pp. 459-500.

Patterson, J. W., M. F. Sudlow and S. R. Walker, Blood-Levels of Fluorinated Hydrocarbons in Asmatic Patients After Inhalation of Pressurized Aerosols. *The Lancet* 2 (7724) 565-568 (1971).

Ullrich, V., and H. Diehl, Uncoupling of Monooxygenation and Electron Transport by Fluorocarbons in Liver Microsomes. *Eur. J. Biochem.* 20, 509-512 (1971).

Table I. COMPOUNDS OF INTEREST

A. Substituted methanes

Halon 1301	Bromotrifluoromethane	$CBrF_3$
Halon 1202	Dibromodifluoromethane	CBr_2F_2
Freon 11	Trichlorofluoromethane	CCl_3F
Freon 12	Dichlorodifluoromethane	CCl_2F_2

B. Substituted ethanes

Freon 116	Hexafluoroethane	CF_3-CF_3
Freon 115	Chloropentafluoroethane	$CClF_2-CF_2$
Freon 114	1,2-Dichlorotetrafluoroethane	$CClF_2-CClF_2$
Halon 2402	1,2-Dibromotetrafluoroethane	$CBrF_2-CBrF_2$
Freon 113	1,1,2-Trichloro-1,2,2-trifluoroethane	$CCl_2F-CClF_2$
Halothane	Bromochlorotrifluoroethane	$CHBrCl-Cf_3$

C. Other

Freon C-318	Octafluorocyclobutane	C_4F_8

Table 2. LEVELS OF HALON 2402 IN RAT TISSUE FOLLOWING EXPOSURE BY INHALATION.

Tissue	Post-inhalation interval (hrs) 0	1-1/2	3	24
Liver	258[a]	5	2	0.28
Lung	44	18	2	0.18
Brain	0.70	2.1	0.78	0.36
Kidney	82	27	23	0.33
Heart	24	2.1	2	1.1
Muscle	73	19	2.8	1.0
Fat	365	469	410	11
Blood	87	7	0.23	0.22

a All values shown are in µg Halon 2402/g tissue.

Table 3. HALON 1301 IN RAT BLOOD FOLLOWING A SINGLE 50-MINUTE EXPOSURE TO A VAPOR CONCENTRATION OF 5% (V/V).

Post-inhalation time (hrs)	Blood level µg/g
0	5.6
0.25	0.62
1.0	0.35
2.0	0.05
4.0	0.07

OXYGEN CONSUMPTION IN MITOCHONDRIA FROM RATS EXPOSED TO HALOCARBONS

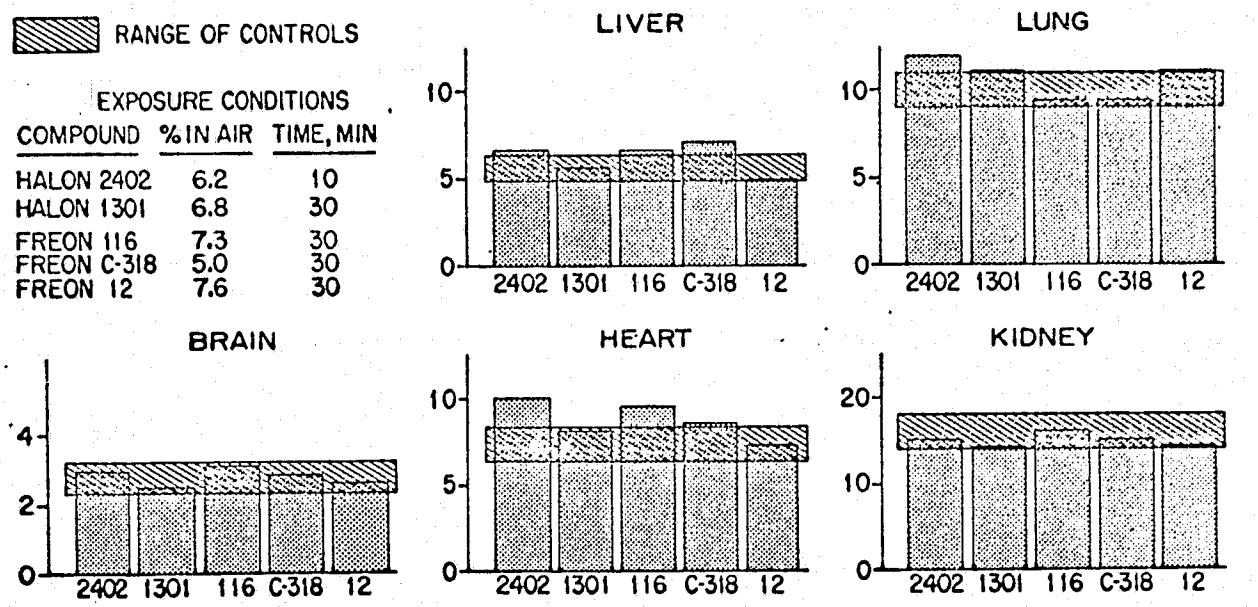

Figure 1. Oxygen Consumption in Mitochondria from Rats Exposed to Halocarbons. Mitochondria were isolated from tissues after the rats were exposed under the indicated conditions. Mitochondria from controls were assayed simultaneously with the experimental groups and the range of activities includes data from all five groups of controls. The rate of oxygen consumption is expressed as mμ AO consumed/mg protein/min X 10^{-1}.

OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM RATS EXPOSED TO HALOCARBONS

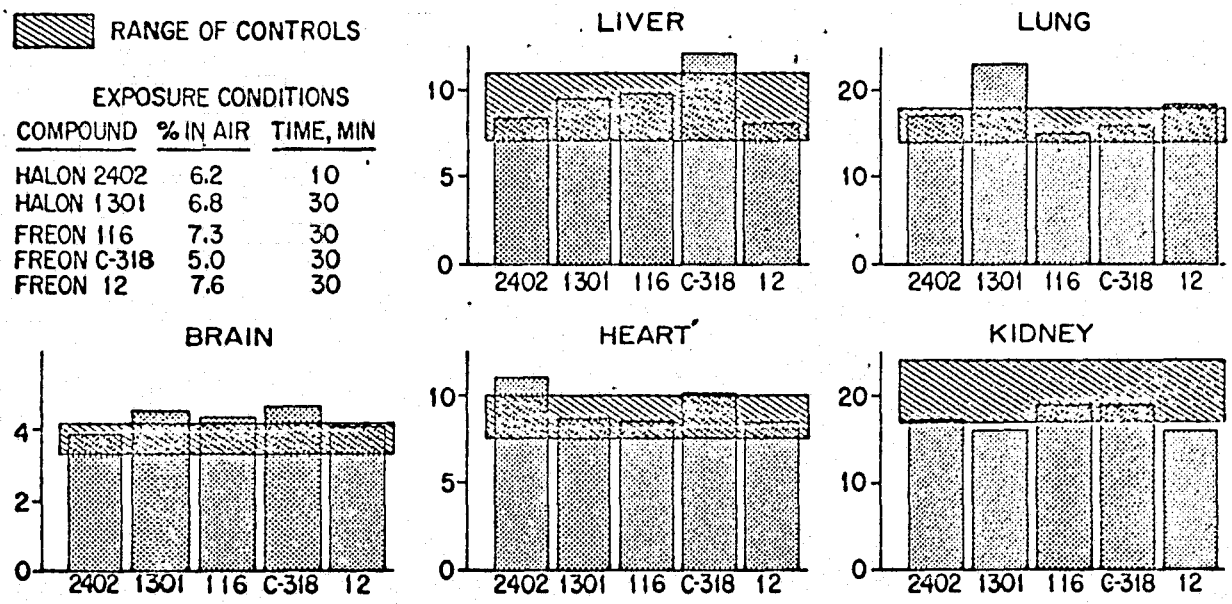

Figure 2. Oxidative Phosphorylation in Mitochondria from Rats Exposed to Halocarbon. Mitochondria were isolated from tissues after the rats were exposed under the indicated conditions. Mitochondria from controls were assayed simultaneously with the experimental groups and the range of activities included data from all five groups of controls. The rate of phosphorylation is expressed as mμ Moles P_i esterified/mg protein/min X 10^{-1}.

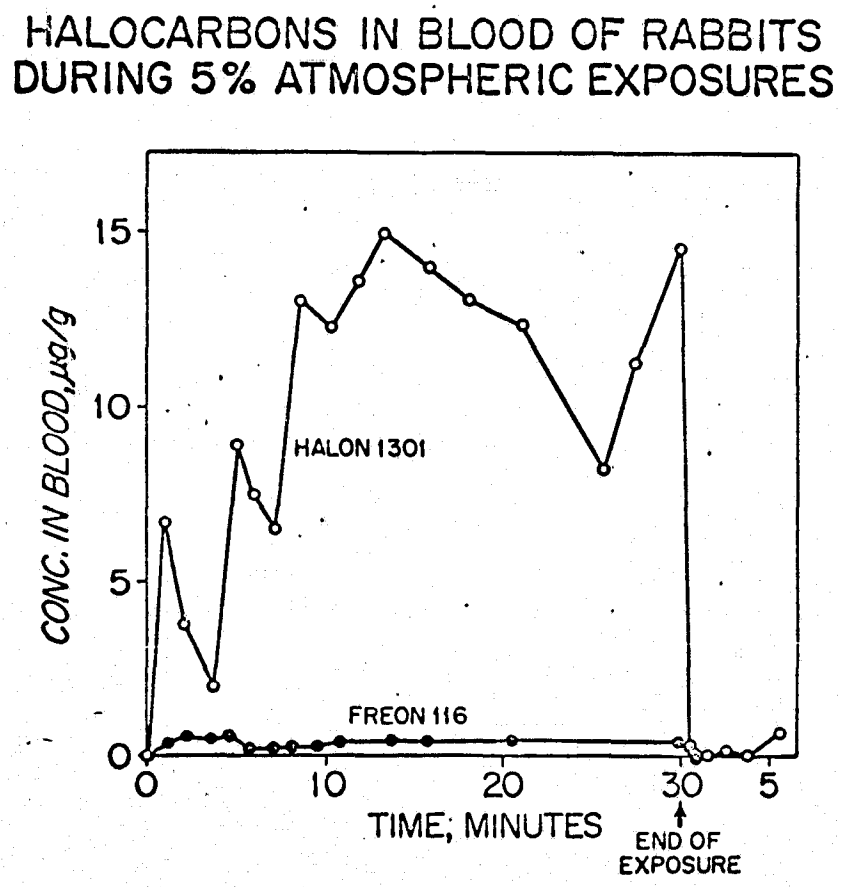

Figure 3. Halocarbons in Blood of Rabbits During 5% Atmospheric Exposures. Blood samples were withdrawn from the animals before, during and after exposures to either Halon 1301 (open circles) or Freon 116 (solid circles. Concentrations of the halocarbons in blood were determined by gas-liquid chromatography.

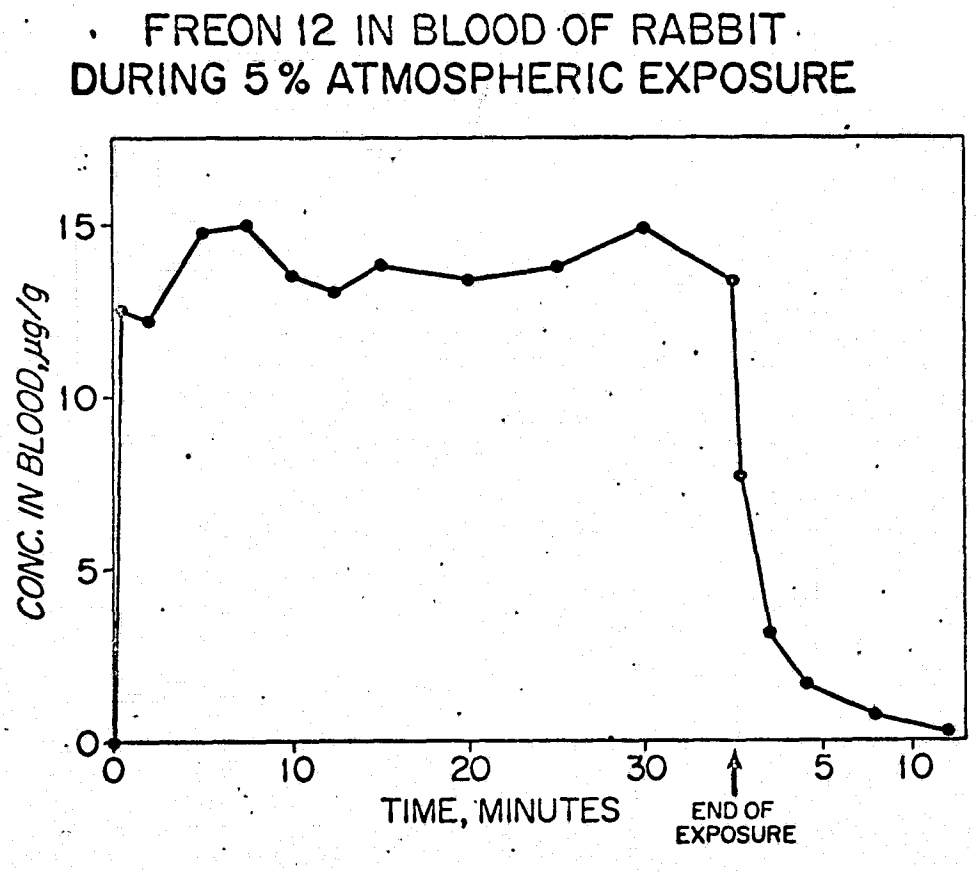

Figure 4. Freon 12 in Blood of Rabbit During 5% Atmospheric Exposure. Blood samples were withdrawn from the animals before, during and after exposure to Freon 12 and the halocarbon concentrations determined by gas-liquid chromatography.

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USE OF ANIMALS IN EXPERIMENTS TO PREDICT HUMAN RESPONSE

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From the standpoint of toxicology, the greatest immediate threat to victims exposed to fire is represented by the smoke and combustion products formed during burning of a variety of materials. In some circumstances, however, there can exist a threat from inhalation of substances other than combustion products, e.g., inhalation of substances used to combat the fire. A number of halogen-containing compounds are being developed as components of fire extinguishers and fire suppressing apparatus and release of these substances contributes to the total toxicologic hazard faced by either victims or persons involved in combatting the fire itself. In fact, as more efficient fire-fighting systems evolve, it is possible that exposure to fire extinguishers and suppressants and their combustion products will constitute more of a hazard than that posed by exposure to combustion products indigenous to the fire itself. In a study of eight fluorinated hydrocarbon compounds that were possible fire extinguishers, Chambers et al. (1950) reported that the pyrolysis products of six compounds were more lethal to rats exposed for 15 min. to atmospheres containing them than the original compounds, in one case by nearly 79 times.

Depending on the nature of the facility to be protected and the type of fire-combatting material employed, there are at least two situations representing rather different toxicologic problems. An acute danger could ensue from the deployment, particularly in a closed space, of a halogenated substance emitted by a fire extinguisher. This could occur in aircraft cockpits or cabins, compartments of ships, warehouses, etc. On the other hand, chronic exposure could follow the use of a halogenated fire suppressant, incorporated into the atmosphere of a vessel, such as a spacecraft, to prevent even the initiation of fire. In this situation, exposure could be continuous for several weeks or even several months.

Both of these problems in toxicology are under investigation in our laboratories and some of the halocarbons we have investigated are shown in Table I. With the exception of Freon C-318 (C_4F_8), all are fluorinated methanes or ethanes. Despite the obvious structural similarities, they produce a variety of pharmacologic effects in laboratory animals ranging from violent convulsions and death following exposure to Halon 1202 (CBr_2F_2) to anesthesia induced by Halothane ($CHBrCl-CF_3$) which is, in fact, a widely used anesthetic mentioned here for comparison purposes only. Others induce myocardial sensitization to catecholamines, e.g. Freon 12 (CCl_2F_2), while others such as Freon 116 (C_2F_6), affect biological systems only subtly if at all. In general, the observations we have made have been in accordance with the generally accepted principle relating the chemical constitution with the toxicity of many of the fluoroalkanes, namely a lower toxicity is associated with an increasing number of fluorine atoms in the molecule (Clayton, 1966). The low order of toxicity in highly fluorinated alkanes is a reflection of low-chemical reactivity and low-biological activity.

Experiments with laboratory animals have shown also that physical chemical characteristics of halocarbons can influence biological activity. For example, blood levels of gaseous compounds such as Halon 1301 ($CBrF_3$) and Freon 116 (C_2F_6) decrease rapidly during the first few minutes after exposure is terminated (Figure 1). Exposure to a less volatile material, such as Halon 2402 (CBr_2F_4), which is liquid at room temperature, leaves substantial residues in the blood (and other tissues) even several hours after the exposure is terminated (Table 2).

One of our objectives has been to determine the extent to which toxicologic studies in laboratory animals can aid in the selection of

halocarbons suitable for long-term continuous exposure in humans inhabiting closed atmospheres containing substantial quantitite of a halocarbon. To aid in the selection, a variety of halocarbons were first studied in short-term exposures to disclose which were the least acutely toxic. The results of such studies have been reported elsewhere (Griffin et al., 1972). One compound, Freon 116, showed a remarkable lack of biological activity in these acute investigations and long-term, continuous exposures were initiated. Freon 116 has also been shown to be potentially useful as a fire suppressant (Huggett, 1973). In our acute studies, Freon 116 was well tolerated by rats and rabbits. No immediate toxic effects were observed even when the concentration of Freon 116 was raised to 20% (v/v) for periods up to one hour. With this background and with the goal of obtaining definitive data during more prolonged exposure, an experiment was devised in which rats would be exposed to the fluoralkane almost continuously for an extended period. We planned an atmospheric concentration of 20% (v/v), an effective concentration of Freon 116 for use as an inerting gas. A concentrated multidisciplinary approach was taken because only a limited number of animals would be available.

Hematological and biochemical serum examinations were selected with a two-fold purpose in mind. In the first place, we wanted data on the general health and clinical status of the animals and this was obtained through a battery of tests performed at the termination of the exposure. Secondly, some selected tests were incorporated with the hope of showing specific sensitivity to exposure to the halocarbon. Osmotic

fragility studies were employed to demonstrate whether the fluorocarbon had any effect on the integrity of the erythrocyte membrane. It was hoped that studies of enzyme activities within serum would disclose liver or other tissue damage.

Preliminary studies employing acute exposures had not revealed lability of the carbon-fluorine bond as determined by measurements of concentrations of fluoride in samples of urine obtained after the exposures. This approach to studying the metabolism of Freon 116 was employed in the present experiment also. We felt also that, since excess fluoride accumulates in bone, a study of that tissue was necessary and would provide a more sensitive indicator of metabolism. Fluoride levels in bone were studied at the termination of the experiment.

Previous biochemical studies had shown that acute exposure to Freon 116 had no effect on mitochondria (Griffin et al., 1972). Since then, the biochemical emphasis has been to determine whether perfluorinated hydrocarbons have any effect on the endoplasmic reticulum of the liver. Mixed function oxidase and cytochrome P-450 were measured to determine whether the Freon 116 would induce production of processing enzymes in the microsomes.

For exposure of the animals, a portable animal exposure chamber as described by Hinners et al. (1968) was modified to operate in a "closed dynamic" mode for control of the gaseous content. This manner of operation was utilized because of long-term exposure studies involving concentrations of Freon 116 of the order of 20% would necessitate the use of large quantities of the fluorocarbon if the open dynamic system of exposure were used.

Such a system would not be economically feasible because of the high cost of the fluorocarbon; therefore, a recirculating system, based on that described by Clayton et al. (1960), permitting the recovery and reuse of Freon 116 was devised. In our variation of the closed bynamic system chamber gases were circulated through a bed of lithium hydroxide to remove carbon dioxide. For each mole of carbon dioxide removed, it was necessary to add back an equivalent amount of oxygen. This was accomplished by metering oxygen to the changer at a rate required to maintain the concentration at about 20%. Freon 116 was also metered to the chamber at the rate required to maintain a 20% by volume concentration.

The chamber was operated for 23 hours a day, 7 days a week. The daily one hour down time was used for animal care and for cleaning. In order to prevent the build-up of ammonia and other odorous materials, strict standards of cleanliness were observed. Animal cages and the trays for collecting urine and feces were changed daily and the inside of the chamber was washed each day. Urine and any spillage from water bottles was absorbed on Pel-E-Cel (Paxton Products). In addition, boric acid crystals were sprinkled on the trays holding the absorbent to help prevent the formation of ammonia. Finally, the chamber was purged at a slow rate throughout the exposure period to help prevent the build-up of foreign materials in the atmosphere.

With the chamber operating in this manner, we determined that its size limited the exposure capability to about 12 rats; therefore, the experiment was started with that number of male Sprague-Dawley rats in the

chamber. Twelve male animals housed outside the exposure chamber served as controls for the study. The exposure lasted for 37 weeks, or nearly 9 months.

The concentrations of Freon 116, oxygen, and carbon dioxide measured during the study are shown in Figure 2. The grand average of the weekly Freon 116 concentrations was 20.7% and the averages of those of oxygen and carbon dioxide were 20.1% and 0.39% respectively. The system of exposure proved to be a suitable one for maintaining the gaseous concentrations at the desired levels, although there were some variations. Carbon dioxide concentrations were maintained well below the upper limit of 1% which would have been tolerated. Lesser variations in the concentration of CO_2 after the 15th week of exposure followed an improvement in the design of the lithium hydroxide scrubber. The temperature within the chamber reflected the temperature outside the chamber except that conditions were 2-3^{0}F higher inside.

The general response of the animals to the exposure conditions is indicated by the body weights shown in Figure 3. Although the weights were equivalent at the beginning of the experiment, growth of the exposed animals was retarded somewhat at the beginning of the exposure and again at about the 18th week of exposure.

Of the original 12 rats admitted to the chamber at the beginning of the exposure, eight survived until the experiment was terminated after 37

weeks of exposure. Two of the animals died during anesthesia for drawing blood samples from the orbital sinus. Two others died from causes which could not be determined from gross examination at autopsy. Autolysis prevented a definitive histopathological examination for determination of the cause of death. Of the twelve animals which entered the study as controls, only six survived, the others dying from respiratory infection.

The general health and well being of the animals is reflected in the hematology data shown in Figure 4. All the parameters examined were well within the normal range as determined in our laboratory, although there was some evidence of hemo-concentration in the exposed rats during the early stages of the experiment. This was indicated by slightly elevated hematocrits and erythrocyte counts in the exposed animals during the 18-week exposure period. However, after 37 weeks of exposure there were no differences between the exposed and the control groups. Total erythrocyte counts were well within the normal ranges at all times and there was no evidence that exposure to Freon 116 elicited a change in the osmotic fragility of erythrocytes. At the termination of the experiment, no differences were observed in the concentrations within serum of glucose, urea nitrogen, sodium or potassium (Figure 5).

With the exception of alkaline phosphatase activity, there were essentially no differences between the exposed and the control groups in the activities of those serum enzymes shown in Figure 6. In the exposed group, the average alkaline phosphatase activity was slightly higher than

that of the controls. This difference was statistically significant ($P<0.05$). LDH activities in the sera of the rats are shown in Figure 7. Significant increases were observed in the exposed rats after 18 weeks of exposure, but this change was no longer present after 37 weeks of exposure to Freon 116.

The biochemical studies of enzymes and levels of cytochrome P-450 in liver (Figure 8) did not show any evidence of the induction common following exposure to many chlorinated compounds.

Concentrations and total daily excretions of fluoride ion in the urines of the rats are shown in Figure 9. Regardless of the manner of expression, the absolute values are slightly lower in the exposed group although there were no significant differences between exposed and control groups of animals. Fluoride ion in the bone of the animals, expressed as per cent of the bone ash, is also shown in Figure 9. There was no significant difference between exposed and control groups.

DISCUSSION

It has been pointed out that the low order of toxicity of highly fluorinated alkanes is a reflection of a low order of chemical reactivity and a low order of biological activity. The experiment described herein confirmed a low order of toxicity of a perfluoroalkane and demonstrated that in spite of the relatively high exposures over a long period of time and under almost continuous conditions of exposure, there was remarkably little response of any kind by the rats that could be attributed to the fluorocarbon. With the chamber containing 20% by volume of Freon 116, the

animals were being exposed by the respiratory route to rather enormous quantities of the chemical. Taking the vapor density of Freon 116 to be 5.7 g/liter and the respiratory minute volume of the rats to be 0.073 liters/min. (Altman and Dittmer, 1964), a conservative estimate for rats weighing 200g or more, it can be calculated that the animals were exposed to about 115g of Freon 116 per day. Certainly, not all was absorbed; in fact, we have shown (Griffin et al., 1972) that rabbits undergoing exposure to 20% Freon 116 had a mean concentration of the Freon in their bloods of less than 1.0µg/g.

The small fluctuations in the activities of serum enzymes, and particularly the elevation of LDH activity during the initial phase of exposure, may have been related to factors other than exposure to the fluorocarbon. In our preliminary studies, in which groups of rats were exposed to fluoroalkanes continuously in the closed dynamic chamber for periods up to 30 days, and in control experiments, in which rats were confined to the chamber for 30 days but were not exposed to a fluoroalkane, elevated LDH activities in serum were often observed. In these experiments, we also noted that slight retardation of growth accompanied confinement of the rats to the chamber and thus did not necessarily result from exposure to a fluoroalkane. It is difficult to conclude what might be the cause of these apparent effects from confinement in the chamber. Their presence within the chamber did expose the rats to an atmosphere different from that of the control animals in more respects than just the presence of Freon 116. As noted above, the temperature was slightly higher and we also observed that the chamber air was often more humid than the air outside. Many closed,

environmentally-controlled chambers incorporate activated charcoal scrubbers and catalytic burners to remove trace amounts of methane, ammonia, odors, etc., from chamber atmospheres, but we could not use such systems because of possible interactions with Freon 116. Hence, the slow purge of chamber gasses was adopted to maintain low levels of such materials, but, even so, minute quantities could still have been present. All of these factors taken together may account for some of the slight differences between control and exposed groups of rats.

Since Freon 116 is a non-polar organic compound and soluble in many organic substances, it is conceivable that the material might be transported to membrane systems and there interact with the lipid components of such systems. It could be anticipated that incorporation of Freon 116 into membranes of cells or components of mixed function oxidase systems might change the integrities of cell membranes or the activities of cellular enzyme systems. If such an interaction occurred in the erythrocyte cell membrane, it was not expressed by a change in the osmotic fragility of the cells.

Decomposition of Freon 116 within the bodies of the rats was not detected by measuring the excretion of the fluoride ion in the urine. Excess fluoride ion accumultes in bone; our failure to find increased concentrations of fluoride in the skeletons of the exposed rats is even stronger evidence that breakage of the carbon-fluoride bond did not occur in these animals. Urinary concentrations of fluoride were low in both the exposed and the control groups and were lower in the exposed group, although by only a small margin; the observed differences were not statistically

significant. However, it is interesting that the trend was lower in the exposed group; this may have been related to differences in dietary intakes of fluoride ion. The growth was slightly retarded in the exposed animals, which may have been due to a decreased consumption of food and a consequent decreased intake of fluoride ion.

Many gaseous hydrocarbons are known to sensitize the myocardium to the effect of catecholamines; although this phenomenon was not studied specifically in our experiment, it is worthy of mention. Studies of this effect of haloalkanes are under study in our laboratories, a preliminary investigation having showed only a low order of catecholamine-induced cardiac arrhythmia caused by Freon 116 (Wills et al., 1972). A comprehensive study of cardiac arrhythmias induced by a variety of halogenated as well as non-halogenated hydrocarbons was reported by Reinhardt et al. (1971), but Freon 116 was not included in this study. In a more recent communication from the same laboratory (Trochimowicz, 1972), Freon 116 at 20% v/v in air was reported not to induce cardiac arrhythmia in dogs. We plan exposures of rhesus monkeys in a study to be conducted in a manner similar to the one reported here for rats but to include also investigations of cardiac sensitivity.

Table I. COMPOUNDS OF INTEREST

A. Substituted methanes

Halon 1301	Bromotrifluoromethane	$CBrF_3$
Halon 1202	Dibromodifluoromethane	CBr_2F_2
Freon 11	Trichlorofluoromethane	CCl_3F
Freon 12	Dichlorodifluoromethane	CCl_2F_2

B. Substituted ethanes

Freon 116	Hexafluoroethane	CF_3-CF_3
Freon 115	Chloropentafluoroethane	$CClF_2-CF_2$
Freon 114	1,2-Dichlorotetrafluoroethane	$CClF_2-CClF_2$
Halon 2402	1,2-Dibromotetrafluoroethane	$CBrF_2-CBrF_2$
Freon 113	1,1,2-Trichloro-1,2,2-trifluoroethane	$CCl_2F-CClF_2$
Halothane	Bromochlorotrifluoroethane	$CHBrCl-Cf_3$

C. Other

Freon C-318	Octafluorocyclobutane	C_4F_8

Table 2. LEVELS OF HALON 2402 IN RAT TISSUE FOLLOWING EXPOSURE BY INHALATION.

Tissue	Post-inhalation interval (hrs) 0	1-1/2	3	24
Liver	258[a]	5	2	0.28
Lung	44	18	2	0.18
Brain	0.70	2.1	0.78	0.36
Kidney	82	27	23	0.33
Heart	24	2.1	2	1.1
Muscle	73	19	2.8	1.0
Fat	365	469	410	11
Blood	87	7	0.23	0.22

a All values shown are in μg Halon 2402/g tissue.

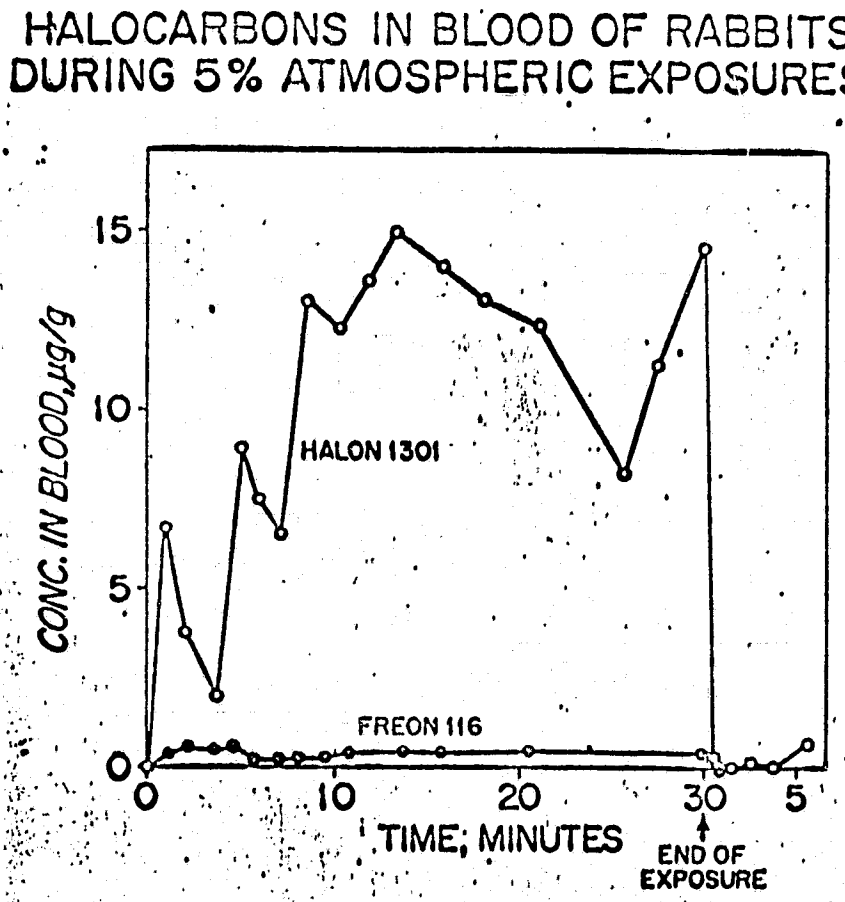


Figure 1. Halocarbons in Blood of Rabbits During 5% Atmospheric Exposures. Blood samples were withdrawn from the animals before, during and after exposures to either Halon 1301 (open circles) or Freon 116 (solid circles. Concentrations of the halocarbons in blood were determined by gas-liquid chromatography.

Figure 2.

CONDITIONS OF EXPOSURE

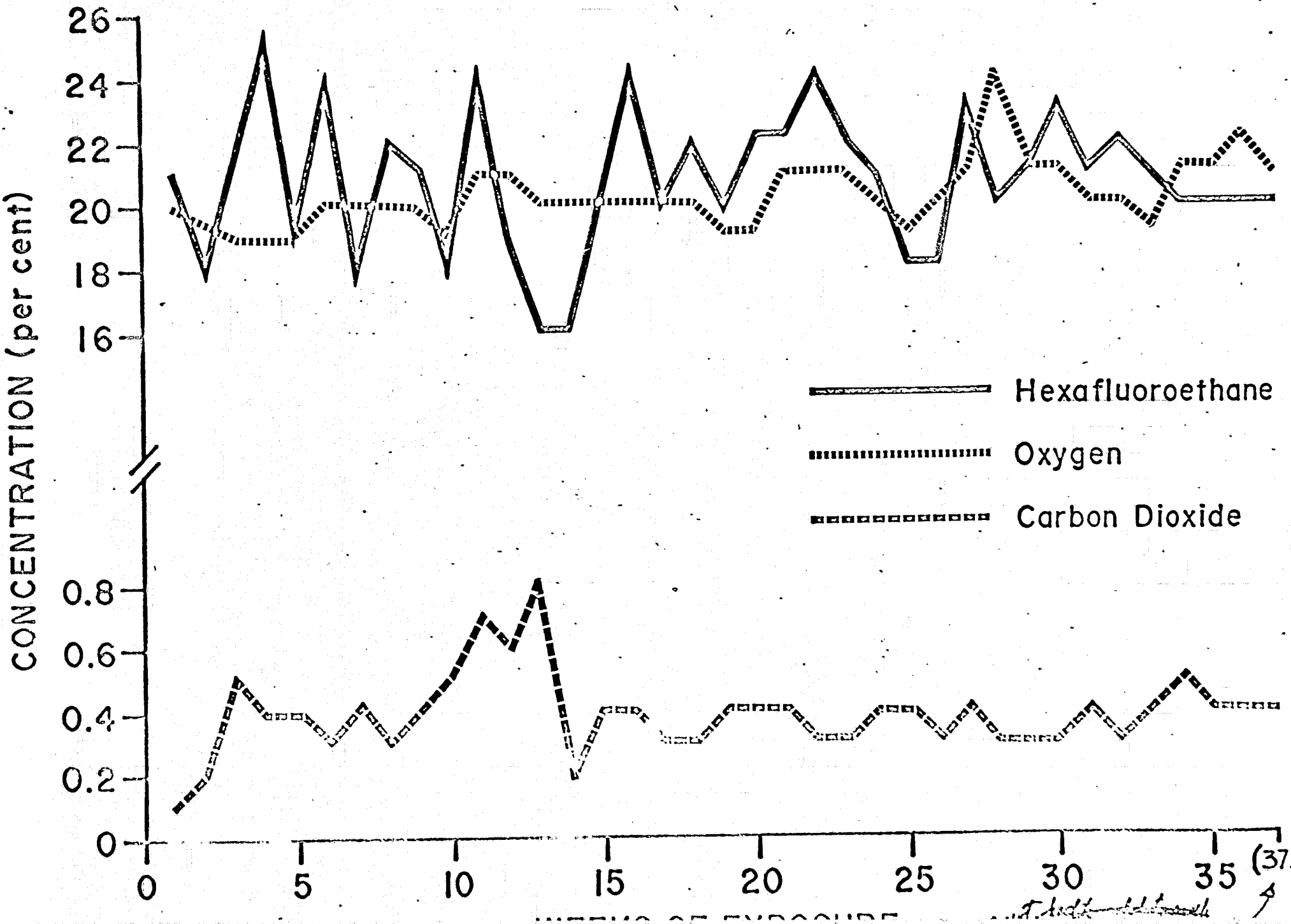


Figure 3.

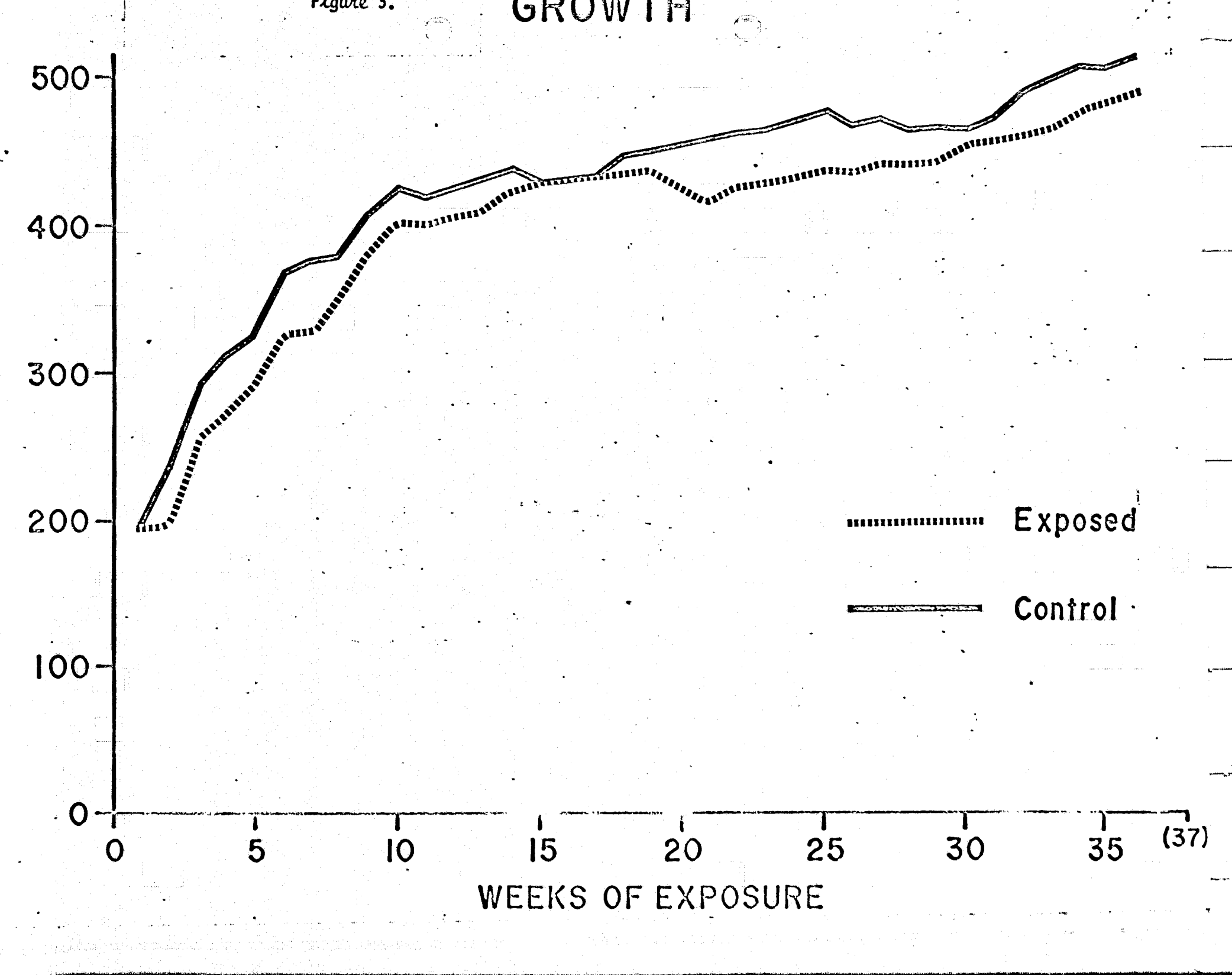
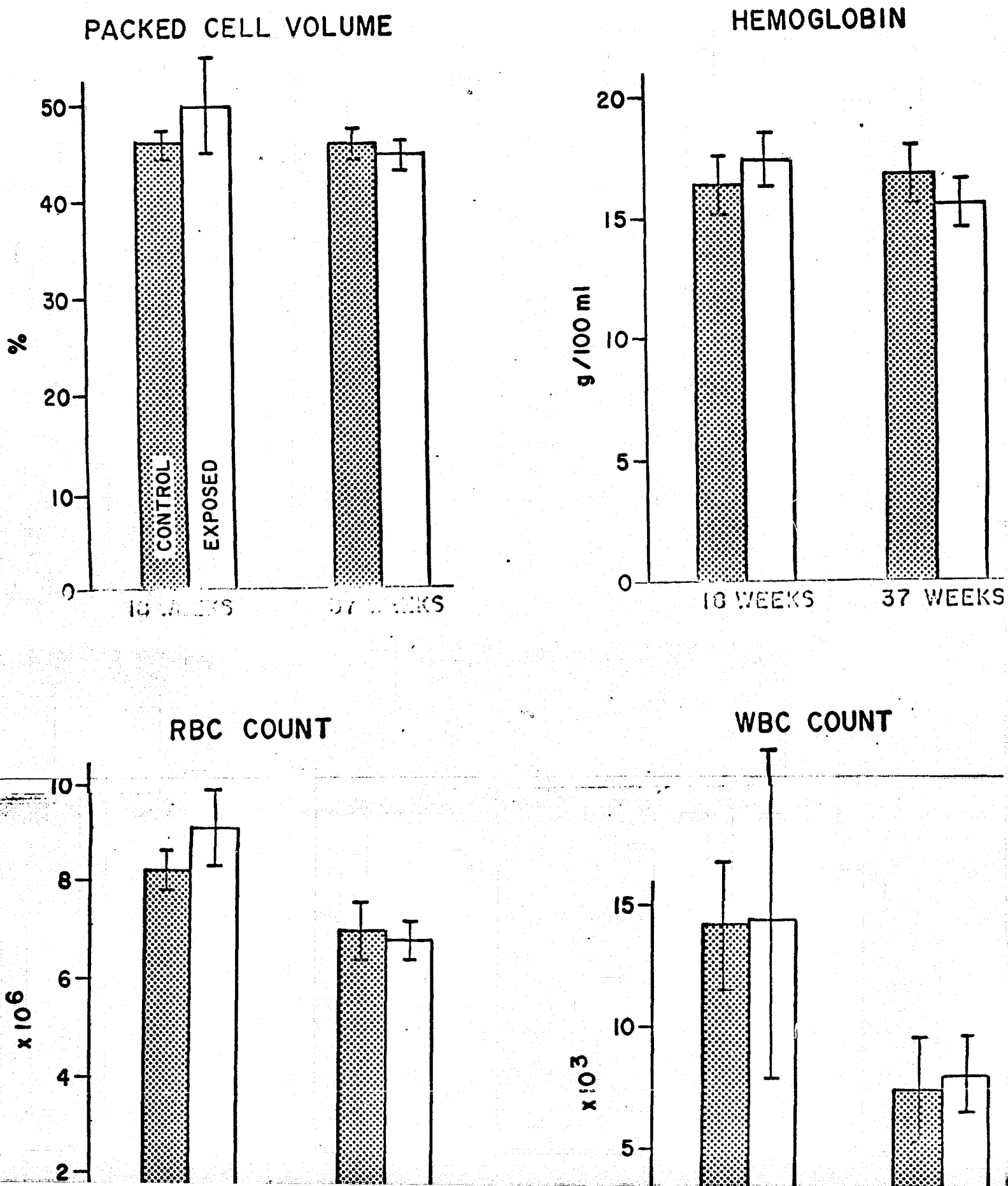
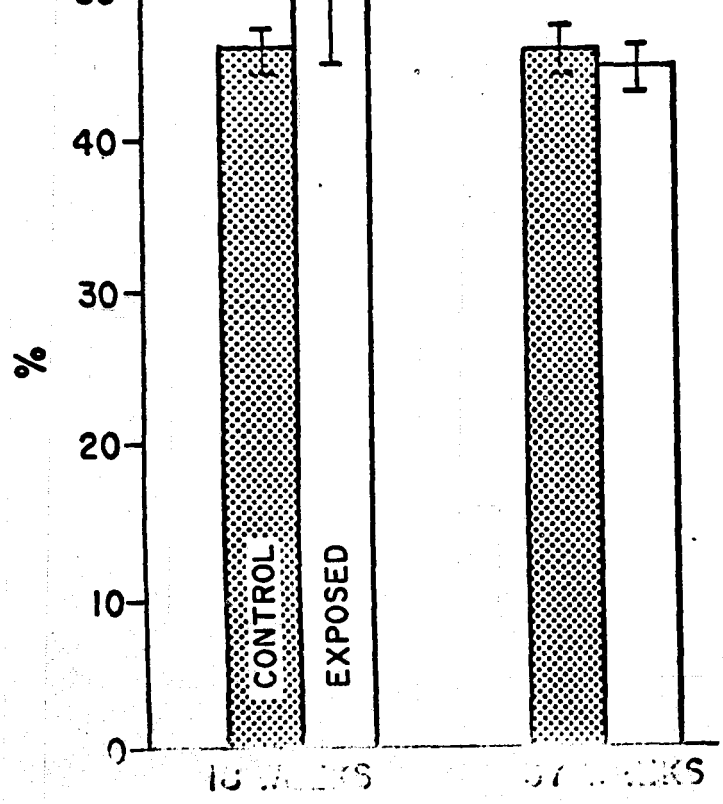
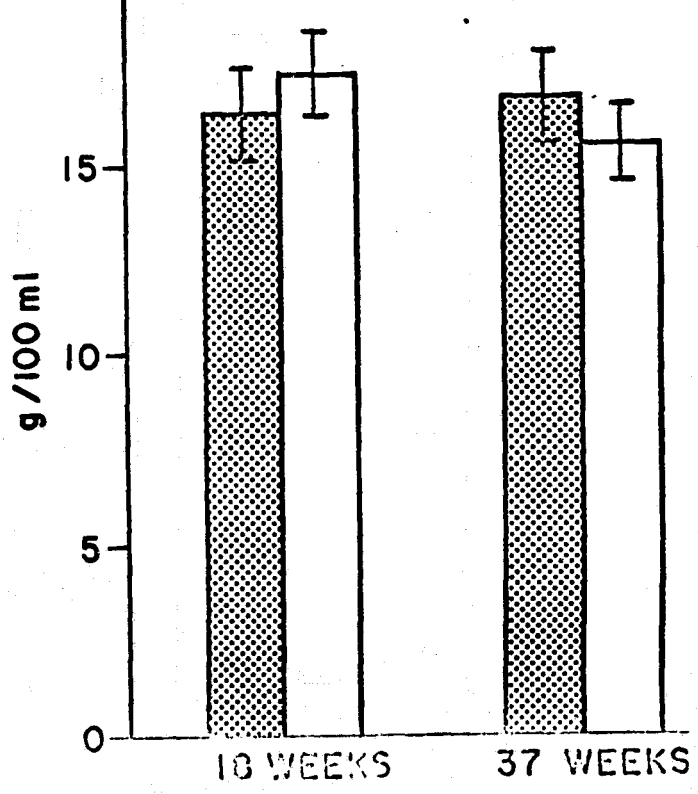

GROWTH
500
400
300
200
100
0
0
5
10
15
20
25
30
35
(37)
WEEKS OF EXPOSURE
Exposed
Control

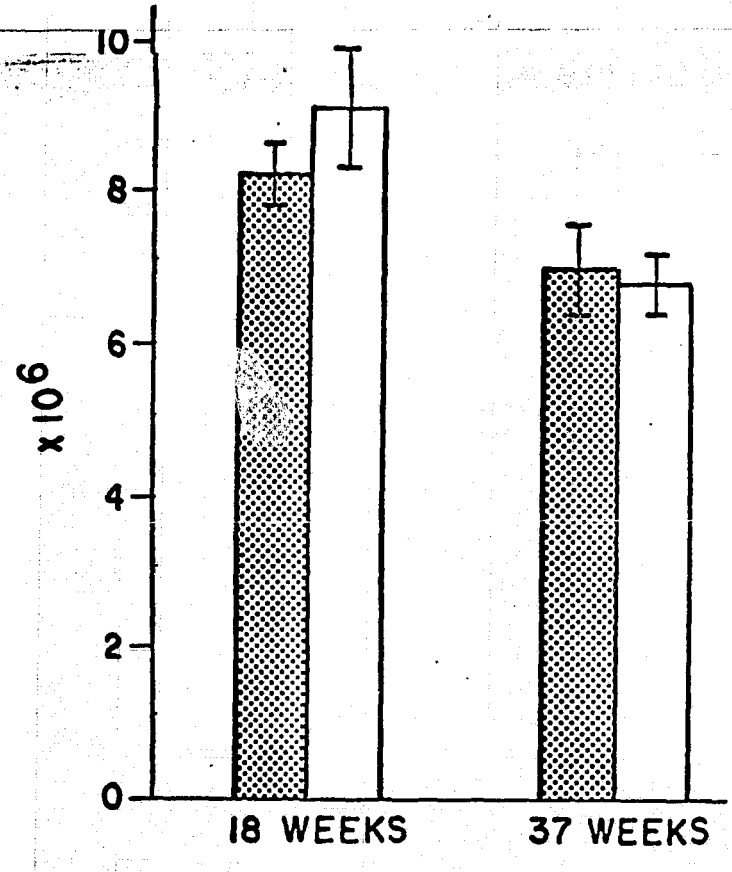
Figure 4.


PACKED CELL VOLUME
%
50
40
30
20
10
0
CONTROL
EXPOSED
HEMOGLOBIN
g/100 ml
20
15
10
5
0
18 WEEKS
37 WEEKS
RBC COUNT
x 10^6
10
8
6
4
2
WBC COUNT
x 10^3
15
10
5

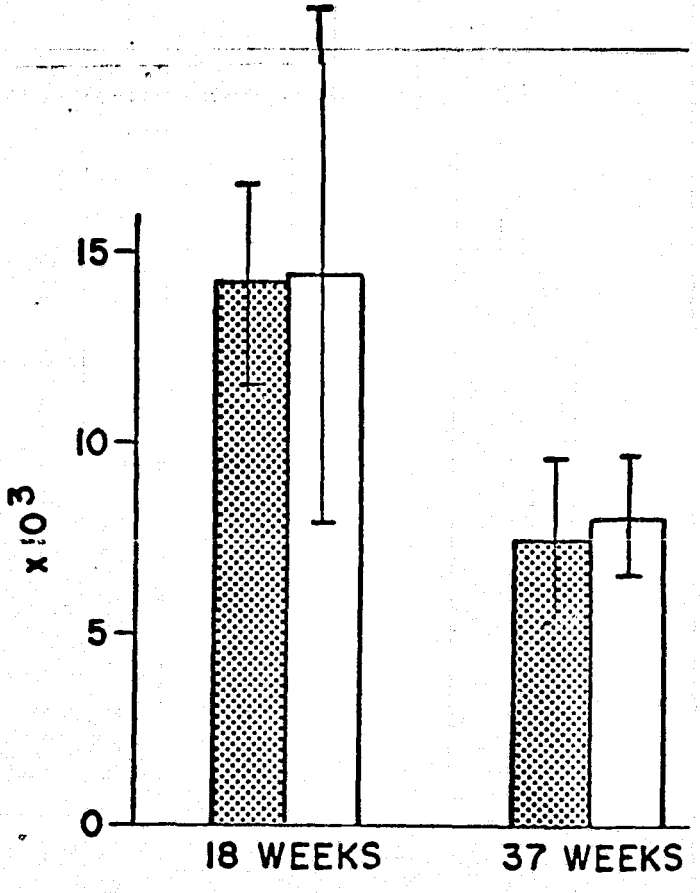




RBC COUNT



WBC COUNT



OLDOUT FRAME 9

Figure 5.

SERUM CHEMISTRY

	CONTROL	EXPOSED
GLUCOSE, mg/100ml	131 ± 16	140 ± 18
UREA NITROGEN, mg/100 ml	18 ± 2	16 ± 3
SODIUM, meq/l	145 ± 2	144 ± 1
POTASSIUM, meq/l	5.1 ± 0.2	5.3 ± 0.4

Figure 6.

SERUM ENZYMES

	CONTROL	EXPOSED
ALK. PHOS, K-A units	14.4 ± 5.9	24.0 ± 7.2
ACID PHOS, K-A units	11.0 ± 2.0	11.1 ± 2.7
GOT, S-F units	65.0 ± 12.0	67.0 ± 21.0
GPT, S-F units	28.0 ± 12.0	23.0 ± 6.0
CPK, units	38.0 ± 24.0	32.0 ± 15.0

Figure 7.

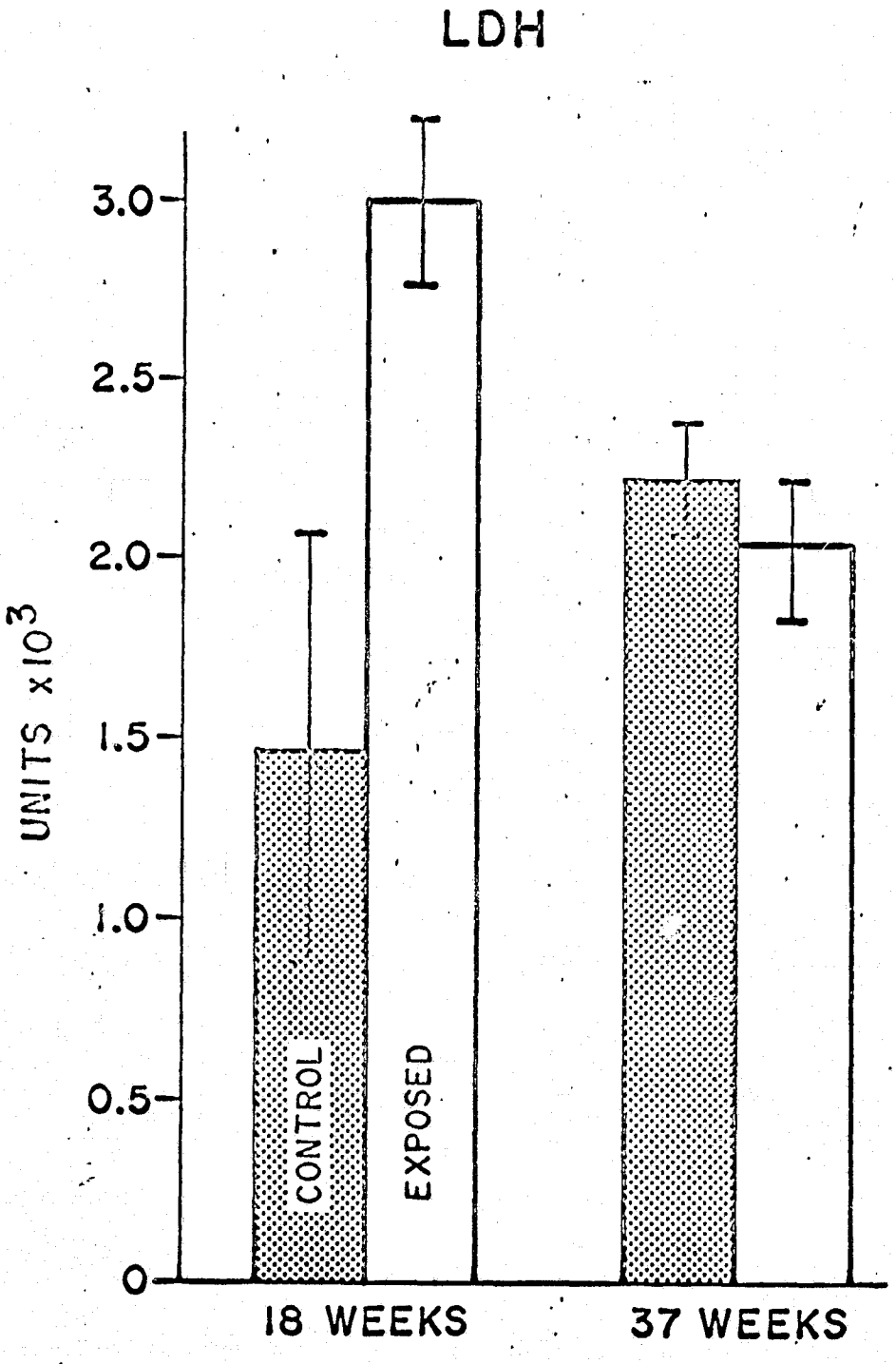

LDH
UNITS x10^3
3.0
2.5
2.0
1.5
1.0
0.5
0
CONTROL
EXPOSED
18 WEEKS
37 WEEKS

Figure 8.

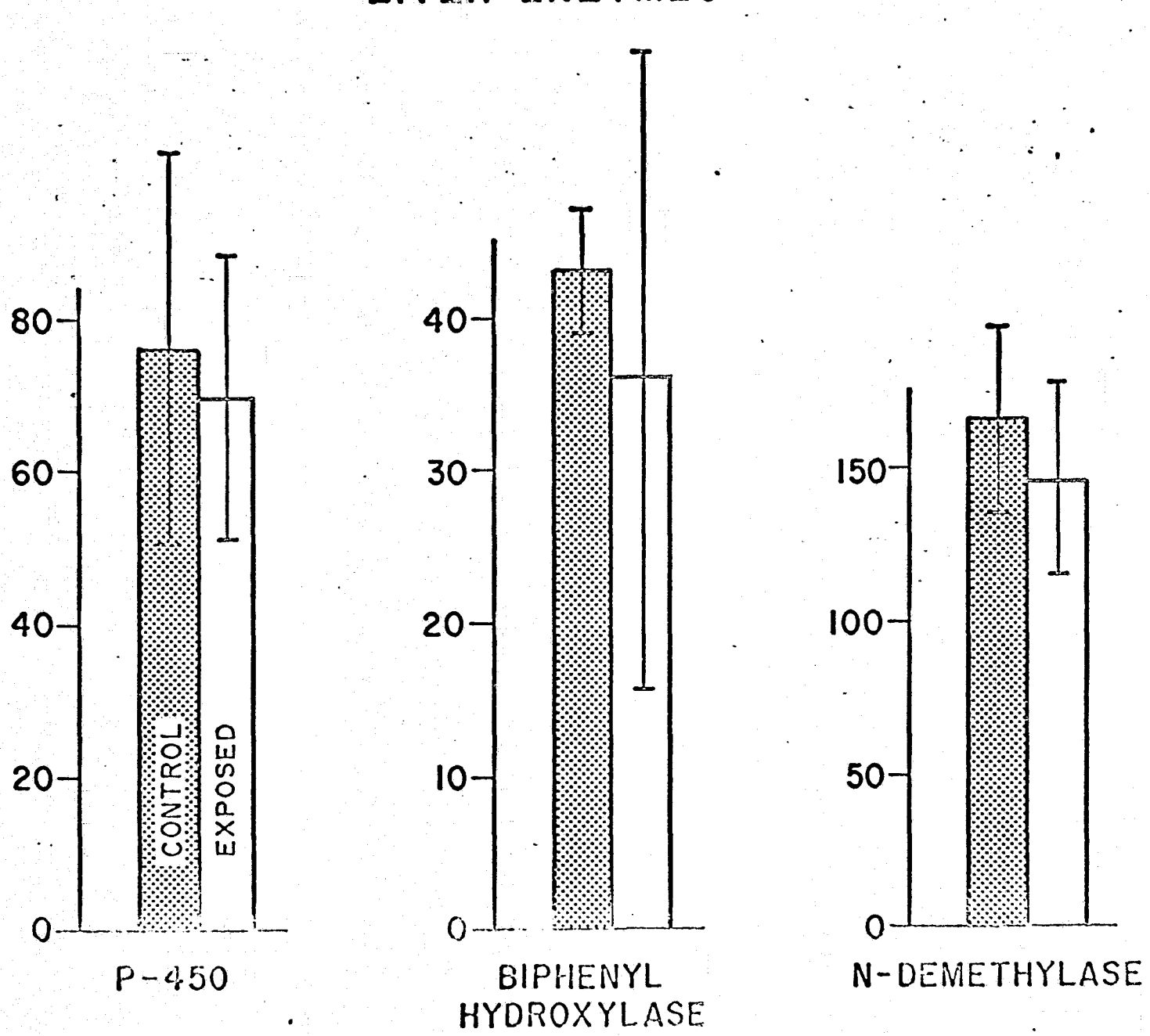
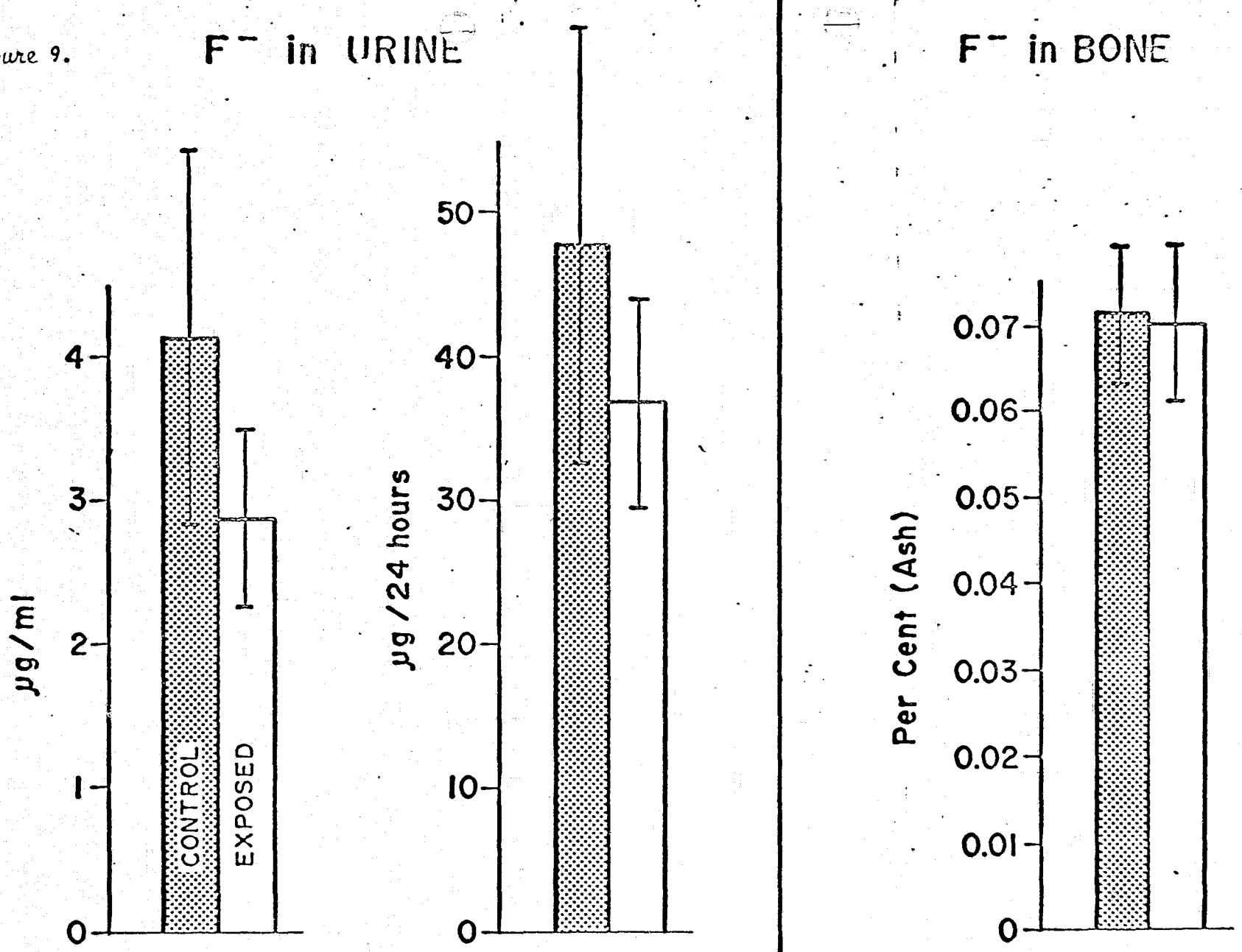

LIVER ENZYMES
80
60
40
20
0
CONTROL
EXPOSED
P-450
40
30
20
10
0
BIPHENYL
HYDROXYLASE
150
100
50
0
N-DEMETHYLASE

Figure 9.


F⁻ in URINE
μg/ml
0
1
2
3
4
CONTROL
EXPOSED
μg/24 hours
0
10
20
30
40
50
F⁻ in BONE
Per Cent (Ash)
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07

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Studies in Rats Exposed Continuously to Hexafluoroethane

by

T.B. Griffin, J.L. Byard and F. Coulston

Among the many applications of short-chain fluorinated hydrocarbons, their current and potential use in combatting and controlling fires in confined spaces promises to be of great benefit in preventing injury and damage. One projected application involves incorporation of a gaseous fluorocarbon in the atmosphere of spacecraft, aircraft, submarines, etc., as an inerting component. Perfluorinated compounds such as tetrafluoromethane, hexafluoroethane and octafluorocyclobutane, have been shown to suppress combustion by increasing the heat capacity of gaseous atmospheres (Huggett, 1973). Before such use of a perfluorocarbon can be initiated, however, much more data on the safety of the compounds for man is required. Although beginnings have been made in studying the toxicology of fluorine-containing hydrocarbons, most investigations have involved only simple exposures of short duration. Little or no data is available relating to the toxic effects which might be encountered during exposure on a continuous basis for a prolonged period. Men occupying spacecraft, aircraft or other confined cabins containing inerting atomospheres would be exposed continuously.

We investigated a variety of fluorocarbons (Griffin, *et al.*, 1972) involving short term single or multiple exposure of laboratory animals. The observations we made were consistant with the generally-accepted principle relating the chemical constitution with the toxicity of many of the fluoroalkanes, namely a lower toxicity is associated with an increasing number of fluorine atoms in the molecule (Clayton, 1966). We determined that, among other compounds, the perfluorinated hydrocarbon hexafluoroethane was well tolerated by rats and rabbits. No immediate toxic effects were observed even when the concentration of hexafluoroethane was raised to 20% (v/v) for periods up to one hour. With this back-

ground and with the goal of obtaining definative data during more prolonged exposure, an experiment was devised in which rats would be exposed to hexafluoroethane almost continuously for an extended period. We aimed at an atmospheric concentration of 20% (V/v), a useful concentration of hexafluoroethane as an inerting gas. A concentrated multidisciplinary approach was taken since only a limited number of animals would be available.

Hematological and serum biochemical examinations were selected with a two-fold purpose in mind. In the first place we wanted data on the general health and clinical status of the animals and this was achieved through a broad-based battery of tests performed at the termination of the exposure. Secondly, some selected tests were incorporated with the hope of showing specific sensitivity to exposure to the halocarbon. Osmotic fragility studies were employed to demonstrate whether the fluorocarbon had any effects on the integrity of the erythrocyte membrane. It was hoped that the studies of serum enzyme activities would disclose liver or other tissue damage.

Preliminary studies employing acute exposures had not revealed lability of the carbon-fluorine bond as determined by measurements of urinary fluoride concentrations. This approach to studying the metabolism of hexafluoroethane was also employed in the present experiment but we also felt that since excess fluoride accumulates in bone, a study of that tissue was necessary and would provide a more sensitive indicator of metabolism. Fluoride levels in bone were studied at the termination of the experiment.

Previous biochemical studies have shown that acute exposure to perfluorinated hydrocarbons has no effect on mitochondria (Griffin, *et al.*, 1972). Since then, the biochemical emphasis has been to determine if

perfluorinated hydrocarbons have any effect on the endoplasmic reticulum of the liver. The very stable carbon-fluorine bonds of perfluorinated hydrocarbons do not appear to be attacked by the mixed function oxidase of the liver endoplasmic reticulum (Ulbrich and Diehl, 1971). However, these authors have found that perfluorohexane binds to mixed function oxidase and stimulates NADPH oxidation. Their findings suggest that exposure to a perfluorinated hydrocarbon might cause the peroxidation of the lipids of the endoplasmic reticulum as a result of a contiuous activation of the mixed function oxidase. To test this hypothesis, lipid peroxides and glucose-6-phosphatase, an enzyme which is sensitive to lipid peroxidative damage, were measured in isolated microsomes. Also, mixed function oxidase and cytochrome b_5 were measured to see if the perfluorinated hydrocarbon would induce the synthesis of microsomal processing enzymes.

METHODS

Exposure of Animals. For exposure of the animals a portable animal exposure chamber as described by Hinners, *et al.*, (1968) was modified to operate in a "closed dynamic" mode for control of the gaseous content. This manner of operation was utilized since long term exposure studies involving concentrations of hexafluoroethane on the order of 20% would necessitate the use of large quantities of the fluorocarbon if a simple flow through system of exposure were used. Such a system would not be economically feasible because of relatively high cost of the fluorocarbon and therefore the recirculating system permitting the recovery and reuse of hexafluoroethane was devised and was based on the system described by Clayton, *et al.*, (1960). In our variation of the closed dynamic system, chamber gases were circulated through a bed of lithium hydroxide (Anhydrous Environmental Grade, Foote Mineral Company) to remove carbon dioxide. The lithium hydroxide was obtained in the anhydrous form, however, water was added at the rate of 750 ml per 10 pounds to improve the efficiency for absorption of carbon dioxide. The concentration of carbon dioxide within the chamber was measured with a Bendix Unico Model 400 gas detector. For each mole of carbon dioxide removed it was necessary to add back an equivalent amount of oxygen. This was accomplished by metering oxygen to the chamber at a rate required to maintain the concentration at about 20%. The concentration of oxygen was monitored by use of a Chemtronics Model GP-10 gas phase oxygen transducer with a Chemtronics Model BP-300A amplifier.

Hexafluoroethane (Freon 116, Du Pont) was metered to the chamber at a rate required to maintain a 20% by volume concentration. The concentration of the fluorocarbon was monitored with a gas chromatograph

(Perkin-Elmer Model 801) having a flame ionization detector and a ¼" x 6' stainless steel column packed with Pora Pak Q (Waters Associates Incorporated). The column bath was maintained at a temperature of 150°C.

The chamber was operated for 23 hours a day, 7 days a week. The daily one hour down time was used for animal care and for cleaning. In order to prevent the build-up of ammonia and other odorous materials strict standards of cleanliness were observed. Animal cages and the trays for collecting urine and feces were changed daily and the inside of the chamber was washed each day. Urine and any spillage from water bottles was absorbed on Pel-E-Cel (Paxton Products). In addition, boric acid crystals were sprinkled on the trays holding the aborbent to help prevent the formation of ammonia. Finally, the chamber was purged at a slow rate throughout the exposure period to help prevent the build-up of foreign materials in the atmosphere.

With the chamber operating in this manner we determined that its size limited the exposure capibility to no more than about 12 rats, and therefore the experiment was started with that number of male Sprague-Dawley rats in the chamber. Twelve male animals housed outside the exposure chamber served as controls for the study. The exposure lasted for 37 weeks or nearly 9 months.

Clinical Chemistry and Hematology. During some of our preliminary studies of rats undergoing exposure to fluorocarbon chemicals for periods up to one month, we oberved increases in levels of serum lactic dehydrogenase (LDH) activity. This was investigated in the present study by measurement of the enzyme activity at two intermediate periods and at the time of the terminal sacrifice. During these intermediate periods the hematocrit, hemoglobin concentration, and red and white total cell counts

were also measured. More frequent sampling was avoided since the necessary anesthesia stressed the animals to an undesirable degree.

At the time of the terminal sacrifice more extensive serum studies included, in addition to measurements of LDH activity, determinations of acid and alkaline phosphatase activities, glutamic-oxaloacetate and glutamic-pyruvate transaminase activities, creatine phosphokinase activity, glucose, urea nitrogen, sodium and potassium. Added to the hematology studies were determinations of differential white cell counts and erythrocyte osmotic fragility.

Measurement of Fluoride Ions. Following the 37 weeks of exposure to hexafluoroethane, concentrations of fluoride were measured in urine samples collected during a 24 hour period. A procedure described for the determination of fluoride ion content of human urine (Singer, *et al.*, 1969) was modified to permit suitable measurements in rat urine. Each volume of urine was diluted with four volumes of a solution containing 8.5 g NaCl and 5 g of disodium ethylenediaminetetraacetic acid (EDTA) per liter. The solution was adjusted to pH 6.5 with dilute potassium hydroxide before dilution to final volume and additon to the rat urine. Standard solutions of fluoride ion were also prepared with the NaCl-EDTA diluent and a standard curve prepared with the fluoride ion selective electrode. In order to obtain another basis for the rate of urinary fluroide excretion, creatinine levels were measured in each of the urine samples.

At the time of sacrifice, a femur from each animal was disected free of soft tissue, dried and defatted with ethyl ether. The bones were then dry ashed at 500^{0}C and determination of fluoride in the bone was made with the fluoride electrode using the procedure of Singer and Armstrong (1968).

Hepatic Biochemistry. NADP, glucose-6-phosphatase and thiobarbituric acid were purchased from Sigma Chemical Company, biphenyl, p-chloroaniline and demethylaminobenzaldehyde were purchased from Eastman Kodak Company, 4-hydroxybiphenyl from Aldrich Chemical Company, and p-chloroaniline from Calbiochem Company. Livers were homogenized in 5 volumes of 0.25 M Tris-HCl pH 7.4. Microsomes were prepared by differential centrifugation. The $0.25\text{-}6\cdot10^6$ g.min pellet was resuspended in 0.25 M sucrose-0.01 M Tris-HCl pH 7.4 for the enzyme assays and in 1.15% KCL for the cytochrome and lipid peroxidation assays. The cytochrome P-450 and b_5 assays were performed on fresh microsomes, while the remaining assays were done on microsomes which had been frozen one time.

Cytochrome P-450 and b_5 were assayed by the method of Mazel (La Du, *et al.*, 1971), biphenyl hydroxylase by Creaven's method (Creaven *et al.*, 1965), N-demethylase by Kupfer's method (Kupfer and Bruggeman, 1966), lipid peroxidation by Wills' method using NADPH (Wills, 1969), and glucose-6-phosphatase by Nordlei and Arions method (Nordlei and Arion, 1966).

Protein was determined by a modified Gornall procedure (Gornall, *et al.*, 1949) adapted to a Technican Autoanalyzer. One tenth ml of 5% sodium dodecyl sulfate and one tenth ml of sample containing 0.5-4 mg protein were mixed in a 0.5 ml micro sampling cuvette. The cuvettes, including those containing buffer and 1,2 and 4 mg of bovine serum albumin were sampled by the autoanalyzer. About 0.06 ml of sample were mixed with 20 volumes Biuret reagent containing 2% NaoH and disodium tartrate in place of sodium potassium tartrate. The mixture was passed through a small mixing coil and then directly into a flow cell colorimeter where the absorption at 550nm was measured. A linear standard curve was obtained as well as highly reproducible determinations of protein in all subcellular fractions, including mitochondria.

RESULTS

General Response to Exposure. The weekly average concentration of hexafluoroethane, oxygen and carbon dioxide are shown for each week of the experiment in Table 1. The average temperature within the chamber during each week is also shown in the table. The grand average of the weekly hexafluoroethane concentrations was 20.7% and the average of oxygen and carbon [illegible] was 20.1% and 0.39% respectively. The system of exposure utilized herein proved to be a suitable one for maintaining the gaseous concentrations at the desired levels although there were some variations. Carbon dioxide concentrations were maintained well below the upper limit of 1% which would have been tolerated. The temperature within the chamber reflected the temperature outside the chamber except that conditions were 2-3^{0}F higher inside.

The general response of the animals to the exposure conditions is reflected in the body weights also shown in Table 1. Although the weights were equivalent at the beginning of the experiment, growth of the exposed animals was retarded somewhat at the beginning of the exposure and again from about the 15th through the 23rd weeks of exposure.

Of the original 12 rats admitted to the chamber at the beginning of the exposure, eight survived until the experiment was terminated after 37 weeks of exposure. Two of the animals died during anesthesia being administered to draw blood samples from the orbital sinus. Two others died from causes which could not be determined from gross examination at autopsy. Autolysis prevented a definative histopathological examination for determination of the cause of death. Of the twelve animals which began the study as controls, only six survived, the remaining having died from an intercurrent respiratory infection.

Serum Biochemistry. The result of biochemical studies of serum obtained from the rats after 37 weeks of exposure to hexafluoroethane are shown in Table 2.

With the exception of the levels of alkaline phosphatase activity there were essentially no differences between the exposed and control groups. In the exposed group the average alkaline phosphatase activities was slightly higher than the controls. This difference was statistically significant ($P < 0.05$).

Levels of LDH activity in the serum of the rats are shown in Table 3. Significant increases were observed in the exposed rats after 18 weeks of exposure but this difference had disappeared by 34 weeks and was not observed at the termination of the experiment after 37 weeks of exposure to hexafluoroethane.

Hematology. Osmotic fragility of the erythrocytes, measured after 37 weeks of exposure, is shown in Table 4. It can be concluded from this data that exposure of the animals to hexafluoroethane did not alter the integrity of the erythrocyte membrane at least as could be determined by a challenge from osmotic shock.

General health and well being of the animals is reflected in the hematology data shown in Table 5. All of the parameters examined were well within the normal range as determined in our laboratory, although there was some evidence of hemo-concentration in the exposed rats during the early stages of the experiment. This was indicated by slightly elevated hematocrit and erythrocycte counts in the exposed animals during the 18 week exposure period. However, after 34 weeks of exposure there were no differences between the exposed and control groups nor were there any differences when the study was termintated. Total erythrocyte counts were well within the normal ranges at all times.

Fluoride in Urine and Bone. Levels of fluoride ion excreted in the urine by the rats as shown in Table 6. Three methods are used in the table to express the rate of fluoride ion excretion. In the first, it is expressed as a simple concentration in the urine, in the second it is expressed as total excretion in the 24-hour period, and in the third it is expressed as amount excreted per unit weight of creatinine excreted. Regardless of the manner of expression the absolute values are slightly lower in the exposed group although there were no significant differences between exposed and control groups of animals. Level of fluoride ion in the bone of the animals, expressed as a per cent of the bone ash, is also shown in Table 6. There was no significant difference between exposed and control groups.

Hepatic Biochemistry. The 37 week exposure to hexafluoroethane had no significant effect on any of the parameters measured (Table 7). Mixed function oxidase was not induced. No evidence of lipid peroxidation, in terms of increased malonaldehyde production or inhibition of glucose-6-phosphatase, was found.

DISCUSSION

It has been pointed out that the low order of toxicity of highly fluorinated alkanes is a reflection of a low order of chemical reactivity and a low order of biological activity (Clayton 1967). The experiment described herin confirmed a low order of toxicity and demonstrated that in spite of the relatively high levels of exposure to hexafluoroethane over a long period of time and under almost continuous conditions of exposure, there was remarkably little response of any kind by the rats. With the chamber containing 20% by volume of hexafluoroethane, the animals were being exposed by the respiratory route to rather enormous quantities of the chemical. Taking the vapor density of hexafluoroethane to be 5.7 g/liter and the respiratory minute volume of the rats to be 0.073 liters/min. (Altman and Dittmer, 1964), a conserative estimate for rats weighing 200 g or more, it can be calculated that the animals were exposed to about 115 g of hexafluoroethane per day. Certainly not all was absorbed and in fact we have shown (Griffin, *et al.*, 1972) that in rabbits undergoing exposure to 20% hexafluoroethane, the concentration in blood was less than 1.0 µg/g.

The small fluctuations in activity of serum enzymes, and particularly the elevation of LDH activity during the initial phase of exposure, may have been related to factors other than exposure to the fluorocarbon. In our preliminary studies in which groups of rats were exposed to fluoroalkanes continuously in the closed dynamic chamber for periods up to 30 days and in control experiments in which rats were confined to the chamber for 30 days but were not exposed to a fluoroalkane, elevated serum LDH activities were often observed. In these experiments we also noted that slight retardation of growth accompanied confinement of the rats to

the chamber and thus did not necessarily result from exposure to a fluoroalkane. It is difficult to conclude what might be the cause of these apparent effects from confinement in the chamber. Confinement in the chamber did expose the rats to an atmosphere different from that of the control animals in more respects than just the presence of hexafluoroethane. As noted above the temperature was slightly higher and we also observed that the chamber air was often more humid than the air outside. Many closed environmentally controlled chambers incorporate activated charcoal scrubbers and catalytic burners to remove trace amounts of methane, ammonia, odors, etc., from chamber atmospheres, but we could not use such systems because of the possible interactions with hexafluoroethane. Hence, the slow purge of chamber gasses was adopted to maintain low levels of such materials but, even so, minute quantities could still have been present. All of these factors taken together may account for some of the slight differences between control and exposed groups of rats.

Since hexafluoroethane is a non-polar organic compound and soluable in many organic substances, it is concievable that the material might be transported to membrane systems and there interact with the lipid components of such systems. It could be anticipated that incorporation of hexafluoroethane into membranes of cells or components of mixed function oxidase systems might change the integrity of the cell membrane or activities of the enzyme systems. If such an interaction occurred in the erythrocyte cell membrane it was not expressed by a change in the osmotic fragility of the cells.

Although perfluorohexane was found by Ulbrich and Diehl(1971) to bind to mixed function oxidase and activate NADPH oxidation, no stimulation of lipid peroxidation or inhibition of glucose-6-phosphatase was found as a result of a chronic exposure to hexafluoroethane. Our negative findings

may be due to the fact that reducing equivalents from NADPH go to H_2O_2 and then to H_2O as suggested by Ulbrich and Diehl instead of going to lipid peroxides. Also hexafluoroethane may not activate P-450 in the same manner as perfluorohexane. The thiobarbituric acid method for measuring lipid peroxides is not very sensitive and measures the tendency of the membranes to peroxidize rather than determining the actual level of peroxides or diene conjugates in the membranes. Although the lipid peroxidation assay is not very sensitive, glucose-6-phosphatase is quite sensitive to lipid peroxidation (Ghoshal and Recknagel, 1965). The fact that this enzyme was not lowered by chronic exposure to hexafluoroethane, indicates that the mixed function oxidase was not activated to produce lipid peroxides.

Decomposition of hexafluoroethane within the bodies of the rats was not detected by means of measurement of fluoride ion excretion in the urine. Excess fluoride accumulates in bone and the lack of evidence of increased levels in bone of the exposed rats is even stronger evidence that breakage of the carbon-fluorine bond did not occur in these animals. Urinary concentrations of fluoride were low in both exposed and control groups and they were lower in the exposed group, although by only a small margin; the observed differences were not statistically significant. However, it is interesting that the trend was lower in the exposed group and this may have been related to differences in dietary intake of fluoride ion. The growth was slightly retarded in the exposed animals and this may have been due to a decreased consumption of food resulting also in a decreased intake of fluoride ion.

Many gaseous halogenated hydrocarbons are known to sensitize the myocardium to the effect of catecholamines and although the phenomenon was not studied specifically in this experiment it is worthy of mention. Studies of this effect of haloalkanes are under study in our laboratories

and a preliminary investigation showed only a low order of catecholamine-induced cardiac arrhythmia caused by hexafluoroethane (Wills, *et al.*, 1972). A comprehensive study of cardiac arrhythmias induced by a variety of halogenated as well as non-halogenated hydrocarbons was reported by Reinhardt, *et al.*, (1971) although hexafluoroethane was not investigated. In a more recent communication from the same laboratory (Trochimowicz, 1972), hexafluoroethane at 20% v/v in air was reported not to induce cardiac arrhythmia in dogs. We plan exposures of rhesus monkeys in a study to be conducted in a manner similar to the one reported here for the rats but also to include investigations of cardiac sensitivity.

TBG*MC
7/10/73

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REFERENCES

Altman, P.L. and Dittmer, D.S., eds., (1964) Biology Data Book, p. 220 Federation of American Socities for Experimental Biology, Washington.

Clayton, J.W., Delaplane, M.A., and Hood, D.B., (1960) Toxicity studies with octafluorocyclobutane. Am. Indust. Hyg. Assoc. J. 21 382-388.

Clayton, J.W. (1966) in Handbuch der Experimentellen Pharmakologie, Vol. XX, pp. 459-500 Eichlen, O., Farah, A., Herken, H., and Welch, A.D., eds., Springer, Berlin.

Clayton, J.W. (1967) Fluorocarbon Toxicity and Biological Action. Fluorine Chem. Rev. 1 197-252.

Creaven, P.J., Parke, D.V., and Williams, R.T. (1965) A fluorimetric study of the hydroxylation of biphenyl *in vitro* by liver preparations of various species. Biochem. J. 96 879-885.

Ghoshal, A.K. and Recknagel, R.O. (1965) On the mechanism of carbon tetrachloride hepatotoxicity: co-incidence of loss of glucose-6-phosphatase activity with peroxidation of microsomal lipid. Life Sci. 4 2195-2209.

Gornall, A.G., Bardawill, C.J. and David, M.M.(1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177 751-766.

Griffin, T.B., Byard, J.L. and Coulston, F. (1972) Toxicological responses to halogenated hydrocarbons. National Academy of Sciences- National Research Council Proceedings of a Symposium on "An Appraisal of Halogentated Fire Extinguishing Agents. pp. 136-147.

Hinners, R.G., Burkart, J.K., and Punte, C.L. (1968) Animal inhalation exposure chambers. Arch. Environ. Health 16 194-206.

Huggett, C. (1973) Habitable atmospheres which do not support combustion Combustion and Flame 20, 140-142.

Kupfer, D. and Bruggeman, L.L. (1966) Determination of enzymetic demethylation of p-chloro-N-methylaniline. Assay of aniline and p-chloroaniline. Anal. Biochem. 17 502-512.

Mazil, P. (1971) in Fundamentals of Drug Metabolism and Drug Disposition pp. 573-575. La Du, B.N., Mandel, H.G., and Way, E.L. eds., Williams and Williams, Baltimore.

Nordlei, R.C., and Arion, W.J. (1966) Glucose-6-phosphatase. Methods in Enzymology 9 619-625.

Reinhardt, C.F., Azar, A., Maxfield, M.E., Smith, P.E., and Mullin, L.S. (1971) Cardiac arrhythmias and aerosol "sniffing". Arch Environ. Health 22 265-279.

Singer, L. and Armstrong, W.D. (1968) Determination of fluoride in bone with the fluoride electrode. Anal. Chem. 40 613-614.

Singer, L., Armstrong, W.D., and Vogel, J.J. (1969) Determination of fluoride content of urine by electrode potential measurements. J. Lab. & Clin. Med. 74 354-357.

Trochimowicz, H.J. (1972) Private Communication.

Ulbrich, V., and Diehl, H. (1971) Uncoupling of monooxygenation and electron transport by fluorocarbons in liver microsomes. Env. J. Biochem. 20 509-512.

Wills, E.D. (1969) Lipid peroxide formation in microsomes. Biochem. J. 113 315-324.

Wills, J.H., Bradley, P., Kao, H., Grace, H., Hull, W., Griffin, T.B., Coulston, F., and Harris, E.S., (1972) Sensitization of the heart to catecholamine induced arrhythmia by haloalkanes. Toxicol. Appl. Pharmacol. 22 305.

Table 1. Weekly chamber conditions and body weights of rats exposed to hexafluoroethane

Week	Chamber conditions				Average Body weight, g	
	Freon, %	O_2, %	CO_2, %	T, °F	Exposed	Control
1	20.8	19.8	0.09	77	194	192
2	18.3	18.1	0.23	77	197	237
3	20.7	19.2	0.51	77	257	290
4	24.8	19.4	0.43	77	271	310
5	18.7	19.4	0.38	77	287	327
6	24.4	19.6	0.34	77	324	366
7	18.4	19.9	0.42	76	326	372
8	22.4	20.0	0.27	77	350	380
9	20.7	19.6	0.39	78	380	408
10	18.3	19.3	0.48	78	396	422
11	24.1	20.7	0.66	77	395	419
12	19.1	20.5	0.58	76	406	422
13	15.6	19.6	0.80	79	409	431
14	16.2	19.9	0.21	79	422	438
15	20.0	19.9	0.43	81	428	426
16	24.0	20.4	0.38	81	434	431
17	20.1	19.9	0.26	81	433	436
18	22.2	19.9	0.29	75	433	447
19	20.2	18.8	0.37	73	436	450
20	21.7	19.5	0.36	77	425	454
21	22.4	20.7	0.39	80	416	456
22	24.3	20.8	0.35	79	426	461
23	21.9	20.5	0.35	77	428	466
24	20.9	20.1	0.42	77	432	470
25	17.8	19.3	0.43	77	435	475
26	18.5	20.3	0.32	77	429	464
27	22.8	20.6	0.41	77	442	470
28	19.6	24.4	0.31	75	439	465
29	20.6	20.7	0.34	76	442	466
30	23.1	20.9	0.33	76	449	462
31	21.3	20.4	0.43	76	455	477
32	21.6	20.1	0.31	76	457	488
33	21.0	19.2	0.35	77	460	499
34	20.2	21.2	0.52	77	466	508
35	20.3	20.5	0.36	77	479	508
36	20.1	21.6	0.42	77	484	509
37	19.7	20.7	0.36	76	489	516

Table 2. Serum biochemistry profiles of rats exposed to hexafluoroethane for 37 weeks

Parameter	Control (N=6)	Exposed (N=8)
Alkaline phosphatase, K-A units	14.4 ± 5.9*	24.0 ± 7.2
Acid phosphatase, K-A units	11.0 ± 2.0	11.1 ± 2.7
Glutamic-oxalacetic transaminase, S-F units	65.0 ± 12.0	67.0 ± 21.0
Glutamic-pyruvic transaminase, S-F units	28.0 ± 12.0	23.0 ± 6.0
Creatine phosphokinase, Sigma units	38.0 ± 24.0	32.0 ± 15.0
Sodium, meq/l	145.0 ± 2.0	144.0 ± 1.0
Potassium meq/l	5.1 ± 0.2	5.3 ± 0.4
Glucose mg/100 ml	131.0 ± 16.0	140.0 ± 18.0
Urea nitrogen mg/100 ml	18.0 ± 2.0	16.0 ± 3.0

*means ± S.D.

Table 3. Lactic dehydrogenase activity in serum of rats exposed to hexafluoroethane

	Units* of Enzyme Activity		
	18 weeks	34 weeks	37 weeks
Control, (N=6)	1470 ± 620	1530 ± 40	2220 ± 150
Exposed, (N=8)	2990 ± 240	1160 ± 270	2050 ± 210

*One unit will reduce 1.0 μmole of pyruvate to lactate per minute at pH 7.5 at 37° C. Values shown are means ± S.D.

Table 4. Osmotic fragility of erythrocytes from rats exposed to hexafluoroethane. Data are expressed as per cent hemolysis.

	% NaCl					
	0.65	0.55	0.50	0.45	0.40	0.30
Control	0	2.8	12.7	56.2	95.1	100
Exposed	0	0	11.5	53.9	94.1	100

Table 5. Hematology profiles of rats exposed to hexafluoroethane.

	Hct %	Hb g%	RBC $x10^6$	WBC $x10^3$
Control, (N=6)				
18 wks	46.5 ± 1.5*	16.4 ± 1.2	8.2 ± 0.4	14.2 ± 2.6
34 wks	48.6 ± 2.1	18.5 ± 1.0	8.2 ± 0.8	8.2 ± 3.0
37 wks	45.9 ± 1.6	16.8 ± 1.2	7.0 ± 0.6	7.5 ± 2.1
Exposed, (N=8)				
18 wks	49.9 ± 5.0	17.4 ± 1.1	9.1 ± 0.8	14.4 ± 6.8
34 wks	48.6 ± 1.9	18.6 ± 1.0	8.3 ± 0.8	8.5 ± 1.4
37 wks	44.7 ± 1.5	15.5 ± 0.7	6.8 ± 0.4	8.1 ± 1.6

*means ± S.D.

Table 6. Excretion of fluoride ion in urine of rats and concentration of fluoride in bone of rats exposed to hexafluoroethane for 37 weeks

	Control (N=6)	Exposed (N=8)
F^- in urine µg/ml	4.13 ± 1.34*	2.83 ± 0.61
F^- excretion via urine µg/24 hr	47.6 ± 15.0	36.6 ± 7.3
F^- excretion via urine µg/mg creatinine	2.65 ± 0.67	2.19 ± 0.45
F^- in bone % bone ash	0.071 ± 0.008	0.070 ± 0.009

*means ± S.D.

Table 7. Biochemical parameters of the microsomal mixed function oxidase in livers of rats exposed 37 weeks to Freon 116

Biochemical parameter	Control (N=6)	Freon (N=8)
cytochrome P-450, $O.D._{450nm} \times 10^3$/mg protein/ml	76 ± 25*	69 ± 18
cytochrome b_5, $O.D._{426nm} \times 10^3$/mg protein/ml	34 ± 14	35 ± 15
biphenyl hydroxylase, $\frac{\text{nMoles 4-OH-biphenyl}}{\text{mg/protein/hr}}$	43 ± 4	36 ± 21
N-demethylase, $\frac{\text{nMoles p-chloroaniline}}{\text{mg/protein/hr}}$	166 ± 30	146 ± 32
lipid peroxidation, $\frac{\text{nMoles malonaldehyde}}{\text{mg/protein/hr}}$	48 ± 12	41 ± 11
glucose-6-phosphatase, $\frac{\mu\text{Moles Pi}}{\text{mg/protein/20 min}}$	3.4 ± 0.9	3.8 ± 1.0

*means ± S.D.

STUDIES OF SENSITIZATION OF CARDIAC MUSCLE TO EPINEPHRINE BY SELECTED HALOCARBON COMPOUNDS

Methods: Guinea pigs and mongrel dogs were anesthetized with sodium pentobarbital, the guinea pigs being injected i.p. and the dogs i.v. Guinea pigs were exposed to the halocarbons by being placed within a plastic static chamber filled with air containing 4 or 5% (V/V) of the halocarbon. Dogs were exposed by breathing from a spirometer through a tracheal cannula a gas mixture containing 4 or 5% (V/V) of halocarbon. With both species, the same general protocol was used:

Time (min.)	Procedure
0	Begin to record ECG.
1-2	Infuse epinephrine i.v. and continue to record ECG.
3-6,7	Continue to record ECG until arrhythmia stops.
7-22	Animal breathes halocarbon.
23	Begin to record ECG.
24-25	Infuse epinephrine i.v. while continuing to record ECG and while animal continues to breathe halocarbon.
26-29,30	Continue to record ECG until arrhythmia stops or animal dies (ventricular fibrillation).

The concentrations of halocarbons used with both the guinea pig and the dog were: 2402-4%; 116, 1301, and C318-5%. The gas mixtures were made by volumetric measure but were not analyzed. They are nominal concentrations, therefore, and are almost certainly higher than would be found by analysis of the gas mixtures. Compound 2402 was found not to yield a stable vapor or aerosol within the spirometer used, so that no actual exposures of dogs to this compound were made.

The effects of the halocarbon compounds on the experimental animals were assessed by comparison of the response to infusion of epinephrine after and during continued inhalation of the halocarbon with that to the similar infusion before exposure to the halocarbon, each animal serving as its own control. In a control experiment, in which two infusions of epinephrine without an intervening exposure to a halocarbon were performed, arrhythmia appeared within 30 and 31.6 seconds after the initiations of the infusions and lasted for 180 and 208 seconds after the cessations of the two-minute infusions.

The responses in the animals exposed to halocarbons were graded on an eight-point scale:

1. Arrhythmia appeared later and lasted for a shorter time than after the control infusion into the same animal.
2. Arrhythmia appeared at about the same time (± 4 sec.) but lasted for a shorter time.
3. Arrhythmia appeared earlier but lasted for a shorter time.
4. Arrhythmia appeared at about the same time (± 4 sec.) and lasted for about the same time (± 4 sec.).
5. Arrhythmia appeared later and lasted for about the same time (± 4 sec.).
6. Arrhythmia appeared later but lasted for a longer time.
7. Arrhythmia appeared at about the same time (± 4 sec.) but lasted longer.
8. Arrhythmia appeared earlier and lasted longer.

Results: The attached seven tables summarize the 41 experiments with guinea pigs and the 12 with mongrel dogs that have been performed. On the basis of the scores assigned according to the scheme given above, 1301 was the least objectionable compound in the guinea pig, followed in order by 2402, 116 and C318. In the dog, C318 was clearly the least objectionable compound, followed by 116 and 1301.

When the scores in the two species are simply summed, 1301 seems to be the least objectionable compound, with C318 following very closely. If man's heart is considered to be more similar to that of the dog than to that of the guinea pig with respect to sensitization to epinephrine by halocarbons, the order of preference of these two compounds would be reversed; in fact, 116 would be considered to be preferable to 1301, falling between C318 and 1301 in preferability.

Conclusions: 1. In the guinea pig, 1301 had the least sensitizing effect on the myocardium, followed in order by 2402, 116 and C318.

2. In the dog, C318 had the least sensitizing effect on the myocardium, followed in order by 116 and 1301.

3. On the basis of the effects of the four halocarbons studied in sensitizing the hearts of the guinea pig and the dog to epinephrine, either 1301 or C318 is judged to be the safest compound for man. The final choice depends upon whether the myocardium of man responds to these halocarbons more as does that of the dog or that of the guinea pig. Orth has stated that he considers the heart of the dog to be the best model of the human heart for such studies.

Table 1. Cardiac sensitization in guinea pigs to Freon 116.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	41	103	155	93	1
2	67	44	171	189	8
3	63	116	118	185	6
4	40	44	152	194	6
5	34	38	135	219	6
6	42	40	203	144	2
7	34	33	149	21	2
8	47	50	48	46	4
9*	22	>120	67	0	5
10	222	55	58	97	8
Mean					5

* No arrhythmia was produced in this animal after exposure to 116.

Table 2. Cardiac sensitization in dogs to Freon 116.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	37	30	213	303	8
2	26	19	206	165	3
3*	29	36	254	272	6
4	28	27	110	18	2
Mean					5

* The spirometer may have been contaminated with 2402 during this experiment; it had been flushed but not cleaned.

Table 3. Cardiac sensitization in guinea pigs to Freon C318.

Animal Number	time to start arrhythmia (Seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	28	47	162	157	5
2	36	39	156	237	6
3	43	53	64	127	6
4	24	52	86	105	6
5	35	42	115	55	1
6	46	48	43	24	2
7	41	52	76	101	6
8*	>120	>120	0	0	4
9	32	78	18	65	6
10	56	37	73	104	8
11	54	52	50	66	7
Mean					5

* No arrhythmia was produced in this animal either before or after exposure to C318.

Table 4. Cardiac sensitization in dogs exposed to Freon C318.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	25	28	207	248	6
2	28	25	204	76	2
3	47	33	144	2	3
4	14	21	262	105	1
Mean					3

Table 5. Cardiac sensitization in guinea pigs to Halon 1301.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	36	46	154	182	6
2*	38	>120	134	0	1
3	26	19	73	228	8
4	38	147	16	33	6
5	36	38	195	36	2
6	33	40	34	28	1
7	28	34	58	21	1
8	37	44	74	6	1
9	40	42	77	92	7
10	38	36	71	67	4
Mean					4

* No arrhythmia was produced in this animal after exposure to 1301.

Table 6. Cardiac sensitization in dogs to Halon 1301.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1	57	78	161	165	5
2	19	34	400	>480	6
3	38	43	195	226	6
4	36	22	176	214	8
Mean					6

Table 7. Cardiac sensitization in guinea pigs to Halon 2402.

Animal Number	time to start arrhythmia (seconds)		duration of arrhythmia (seconds)		Grade
	Before exposure	After exposure	Before exposure	After exposure	
1*	>120	36	0	199	8
2	29	35	92	95	5
3	50	37	162	65	3
4	42	54	45	11	1
5	48	37	58	5	3
6	40	38	124	8	2
7	40	38	37	7	2
8	38	51	141	188	6
9	48	63	76	28	1
10	49	41	65	136	8
Mean					4

* No arrhythmia was induced in this animal before exposure to 2402.

AMRL-TR-72-130

PAPER NO. 16

SENSITIZATION OF THE HEART TO CATECHOLAMINE - INDUCED ARRHYTHMIA

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This work was undertaken as a part of a cooperative effort between the Institute of Experimental Pathology and Toxicology of Albany Medical College and the Manned Space Flight Center of NASA, to find a fire-suppressant, or at least a fire-retardant, that can be present within the atmosphere of a space vehicle without significant hazard to the occupants. A group of fluorinated halocarbon compounds was selected for further study on the basis of the existing information on their antipyral and toxic activities.

The specific task of my group was to determine the effects of candidate chemicals on the cardiovascular system. In view of previous work showing that such halogenated compounds as chloroform (Levy and Lewis, 1911; Brow et al., 1930; Morris et al., 1953; Raventos, 1956), halothane (Raventos, 1956; Hall and Norris, 1958; Katz et al., 1962; Flacke and Alper, 1962), methoxyflurane (Bamforth et al., 1961), and other fluorinated halocarbons (Taylor and Harris, 1970; Reinhardt et al., 1971) are capable of sensitizing the heart to catecholamines, study of the effects of the candidate compounds on the response of the heart to infused catecholamines seemed particularly pertinent.

Methods

Two sorts of studies with guinea pigs, cats, and dogs anesthetised with Na pentobarbital were performed. In both, the animals have been made to breathe a mixture of a halocarbon compound and air, made up in a calibrated spirometer, through an intake-output valve system attached to a cannula inserted into the trachea just below the larynx. In one type of experiment, represented schematically in figure 1, epinephrine was infused into the femoral vein of one hind leg at a rate of about 3.5 μg/kg/min during periods of two minutes before and after exposure to a halocarbon compound during 15 minutes, inhalation of the halocarbon continuing during the second infusion. The second type of experiment was similar except that the infusions of epinephrine were replaced by intravenous injections into the femoral vein of one hind limb of 5, 10, or 15 μg/kg of epinephrine before and at various times after inception of exposure to a halocarbon.

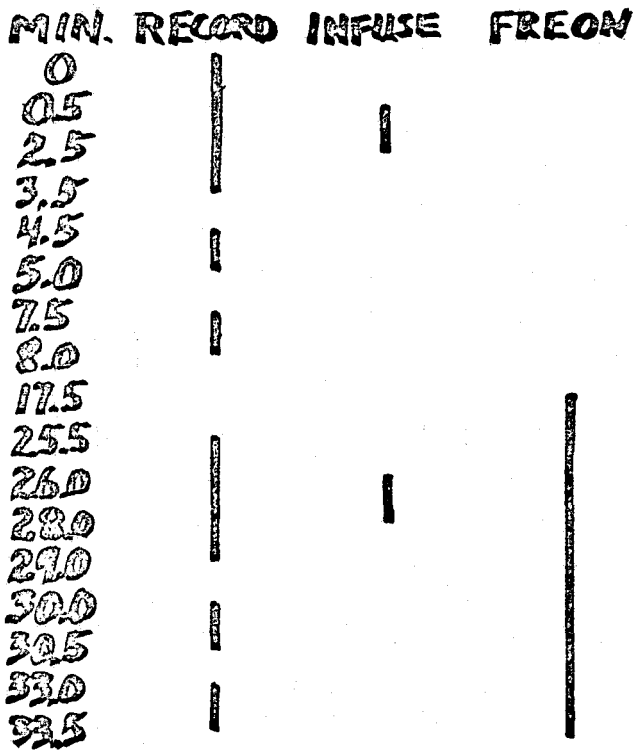

Figure 1. SCHEMATIC REPRESENTATION OF THE SCHEDULE FOR A TYPICAL EXPERIMENT WITH TWO INFUSIONS OF EPINEPHRINE, INDICATING THE TIMES AT WHICH RECORDINGS OF THE ECG (IN SOME CASES OF BLOOD PRESSURE ALSO), INFUSIONS OF EPINEPHRINE, AND INHALATION OF VAPORS OF A HALOCARBON COMPOUND DILUTED IN AIR WERE PERFORMED.

The halocarbon compounds studied were: I = Freon® 11 = trichloro fluoromethane, II = Halon® 1301 = trifluorobromoethane, III = Halon® 2402 = 1,2-dibromotetrafluoroethane, IV = Freon® 116 = 1,2-hexafluoroethane, and V = Freon® C-318 = cyclic octafluorobutane. Some studies have been made also with Freon® 12 = difluorodichloromethane. Halocarbon-air mixtures have been analyzed by gas-liquid chromatography.

Calcium and potassium have been estimated by flame photometry. Norepinephrine has been estimated by the method of Lund (1950), using extraction with perchloric acid, adsorption on alumina, and oxidation with potassium ferricyanide instead of manganese dioxide.

Magnesium chloride, calcium chloride, and MK-486 [L-2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl) propionic acid] have been injected or infused intravenously. The MK-486 was provided kindly by Alexander Scriabine, M. D., of Merck Sharp and Dohme.

Results

Figure 2 shows the responses of a dog to duplicate infusions of epinephrine before and after a 15-minute period of inhalation of a 0.87% (v/v) concentration of Freon® 11 in air, the times indicated beneath the various sections of record being the times elapsed since the start of the infusion. The upper record represents limb lead I in each pane and the lower record limb lead II. The only effect of the infusion in the unexposed animal (left) was some slowing of the heart's rate, whereas in the same animal after exposure to the Freon® compound the duplicate infusion of epinephrine produced initially a more marked slowing of the rate and then the appearance of ectopically generated ventricular contractions and fairly marked arrhythmia. The exposure to the halocarbon compound of itself had little effect on the ECG recordings.

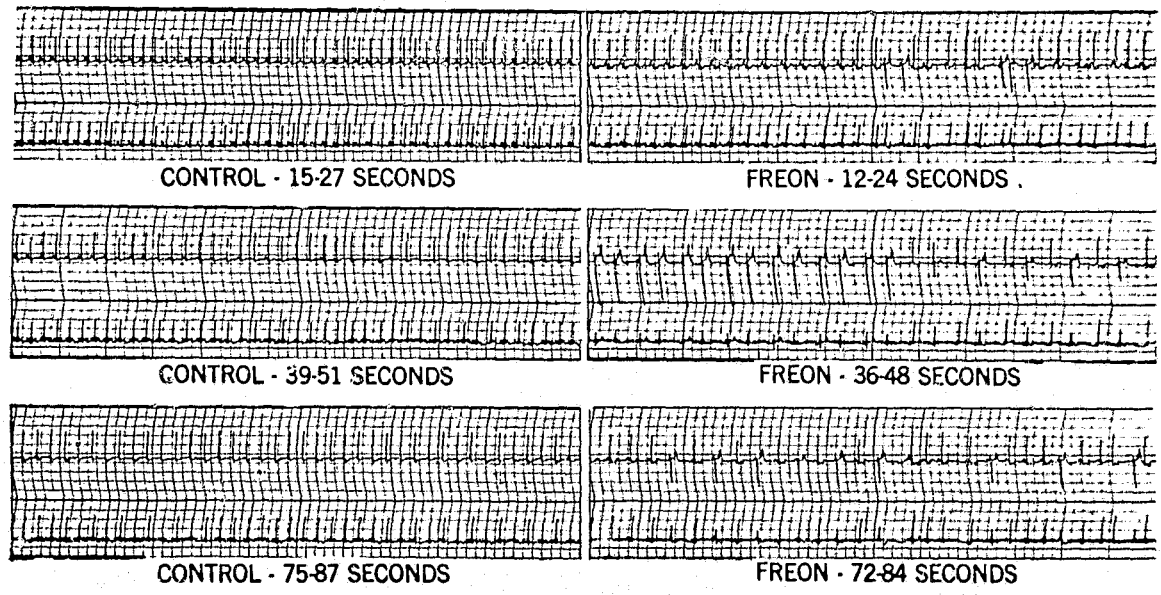

Figure 2. ECG RECORDS MADE AT VARIOUS TIMES AFTER THE BEGINNINGS OF INFUSIONS OF EPINEPHRINE BEFORE (LEFT) AND AFTER (RIGHT) EXPOSURE OF THE ANIMAL TO A VAPORIZED HALOCARBON COMPOUND BY INHALATION.

When the various halocarbon compounds were compared in the type of experiment illustrated above, the results set forth in table I were obtained. The experiments with II and IV in the guinea pig show that although the sensitizing effect of exposure to a halocarbon compound is dose related, the relationship is far from a proportional one; quadrupling the concentration of halocarbon produced only a little more than an eleven percent increase in the number of animals showing sensitization to the infusion of epinephrine, on the average between compounds II and IV. In the case of compound IV, this change in the concentration of the halocarbon compound produced less than a 35 percent increase in the number of animals showing sensitization. This table shows also that the dog and the guinea pig are not entirely identical in their responses to the various halocarbon compounds. Freon® 11 (I) is clearly the most potent of the five chemicals within this table. It is probably followed in order of decreasing effectiveness as a sensitizer to catecholamines by V, II and IV, and III, although the choice among the last four compounds on this basis is not clear.

Table I

Effect of Inhalation of Halocarbon Compounds on Arrhythmia Induced by Infusion of Epinephrine

Compound	Concentration in Air (v/v)	Arrhythmia Increased Guinea Pig	Dog
I	0.87%	6/6	
II	2.2	4/10	3/4
	8.7	2/6	
III	1.8	3/10	1/4
IV	2.2	5/10	2/4
	8.7	2/3	
	33.8	2/2	
V	2.2	7/11	1/4

C. 3

In one cat, exposure to Freon® 11 in a concentration of 1.7% produced arrhythmia without infusion of epinephrine. In a similar experiment in which 8.8% Freon® 12 was administered, spontaneous arrhythmia appeared also. Two cats responded to this concentration of Freon® 12 in this way. These three experiments are the only ones in which arrhythmia has appeared without infusion of epinephrine, although much higher concentrations of some halocarbon compounds have been used [for example, 44% (v/v) of Freon® 116 (IV)]. A comparison of Freons® 12 and 116 (IV) and Halon® 1301 (II) in concentrations of 2.6% (v/v) in the inspired air disclosed that Freon® 12 sensitized more than II to the hypertensive and pulse-pressure elevating effects of infused epinephrine, but less to its chronotropic action. Freon® 12 sensitized less than IV to the hypertensive, pulse-pressure elevating, and chronotropic actions of the infusion of epinephrine. Freon® 12 under these conditions induced arrhythmia during infusion of epinephrine in 1/6 cats whereas the other two halocarbon compounds produced arrhythmia in 0/6 animals.

In experiments in which discrete doses of epinephrine were injected intravenously at various times after beginning exposure to 0.87% (v/v) I, the maximum arrhythmia was found to occur at around 10 minutes of exposure to this most potent of the halocarbon compounds studied; thereafter, despite continued exposure to I, the response decreased. When the exposure to I was terminated, the enhanced responsiveness to epinephrine disappeared rapidly (table II, +5 min. = 5 minutes after termination of the exposure at 15 minutes after its start).

Table II

RESPONSES TO I.V. DOSES OF EPINEPHRINE AT VARIOUS TIMES (MINUTES) AFTER BEGINNING EXPOSURE TO 0.87% (V/V) I

DOSE OF EPINEPHRINE	RESPONSE*				
(μg/kg)	0 MIN.	5 MIN.	10 MIN.	14 MIN.	+5 MIN.
5	4	29	31	21	4
10	30	42	88	28	21
15	73	111	153	123	33

* NUMBER OF ARRHYTHMIC HEART BEATS AFTER EPINEPHRINE.

Because of the well-known interaction of anoxia and epinephrine in the genesis of arrhythmia (see, for example McNamara et al., 1952), a series of animals was given a standard concentration of I (0.87%) made up in air enriched by either nitrogen or oxygen. Sensitization to injected epinephrine was enhanced by low oxygen and decreased, but not abolished, by high oxygen. Section of the vagi, vagal activity being known to enhance the effect of anoxia (McNamara et al., 1952), also reduced, but did not remove entirely, the sensitizing action of I on the chronotropic effect of epinephrine (table III).

Table III

EFFECT OF VAGOTOMY ON THE NUMBER OF ARRHYTHMIC HEART BEATS INDUCED BY I.V. EPINEPHRINE AT VARIOUS TIMES DURING EXPOSURE TO 0.87% (V/V) I

DOSE OF EPINEPHRINE	CONTROL				VAGOTOMISED			
	0 MIN.	5 MIN.	10 MIN.	14 MIN.	0 MIN.	5 MIN.	10 MIN.	14 MIN.
5 µg/kg	2	11	8	0	8	11	7	2
10	60	28	72	0	60	17	6	5
15	116	116	144	104	116	21	9	7

In attempting to identify the change in the heart elicited by exposure to a halocarbon compound, we have used Freon® 11 (I) as the most potent representative of its general chemical type. Whether it is a typical representative remains to be seen, although we have some indication that it is not too different from the others in its qualitative actions.

One obvious factor in sensitization of the heart to extraneous catecholamines is the intrinsic concentration of norepinephrine. To study the effect of exposure to halocarbon compounds on this possible variable, we exposed anesthetised guinea pigs to I and IV by inhalation through a tracheal cannula during a period of 15 minutes. At the end of that time, the animals were killed quickly by intravenous injection of an overdose of Na pentobarbital; the hearts were excised rapidly and put immediately into previously cooled beakers held in an ice bucket. The hearts were homogenized in an all-glass homogenizer and analyzed for norepinephrine. Control animals were treated similarly except that they inhaled normal room air. The results are given in table IV, where each mean represents a group of 6 guinea pigs. Although the concentrations of the two halocarbon compounds used were both known from previous work to be capable of sensitizing the heart of the guinea pig to infused epinephrine, the hearts from the animals exposed to the halocarbon materials did not differ significantly in catecholamine concentration from those from the control animals.

Table IV

Effect of Inhalation of Freon in Air on the Concentration (μg/gm) of Norepinephrine within the Heart of the Guinea Pig

Freon	Concentration	Control	Experimental
116	5.0%	2.29 ± 1.04	2.19 ± 0.81
11	0.4%	1.60 ± 0.36	1.89 ± 0.60

To study the possibility that dopamine contributes to the sensitization of the myocardium by halocarbon compounds, cats were given i.v. doses of 25 mg/kg of MK-486 five minutes before the beginning of exposure to I. This treatment did not modify the response of the heart to infusion of epinephrine at the end of the 15-minute exposure. This result is in sharp contradistinction to those obtained with MK-486 in arrhythmia induced by the administration of DOPA (Mars and Krall, 1971).

Another possible factor in the sensitizing action of the halocarbon compounds is alteration of the relation of the heart to potassium. We have determined that our standard 15-minute exposure to I (in a v/v concentration in air of 0.4%) does not alter significantly the concentration of potassium within blood plasma (table V). We are in the process of determining the effect of this treatment on the concentration of potassium within the heart muscle, but do not have final figures yet.

Table V

Effect of Inhalation of Freon® 11 in Air on the Concentration (meq/liter) of Potassium in Plasma of the Cat

	Control	After Freon
	4.1	4.1
	6.4	4.8
	6.2	4.6
	4.2	4.3
	3.7	3.8
	6.6	5.2
	4.6	4.0
Mean	5.1 ± 1.2	4.4 ± 0.5

We have examined also the effect of i.v. injection of $MgSO_4$ (6 ml/kg of 3.3%) on sensitization of the heart to epinephrine by I, with the result that this dose of $MgSO_4$ did not alter the sensitization. This finding suggests that the halocarbons, unlike digitalis glycosides (Langer and Serena, 1970; Seller, 1971), do not facilitate arrhythmogenic activity through shifting intracellular potassium into the plasma. If the analyses of heart muscle for potassium corroborate this tentative conclusion, we think that we will have good evidence that alteration of the permeability of the myocardial membrane to potassium is not a factor in sensitization of the heart to catecholamines by halocarbon compounds.

We have just begun to study the possibility that the relationship of the myocardial membrane to calcium may be modified by exposure to a halocarbon compound. About all that I am in a position to say with fair certainty at present is that by infusing $CaCl_2$ into cats at a rate of 5 mg/kg/min we have been able to obtain about as great sensitization of the heart to infused epinephrine as we can obtain by the standard exposure to I. A different sort of experiment will be undertaken soon to attempt to determine more directly whether calcium is involved in sensitization by halocarbon compounds, possibly by enhancing release of catecholamines from secretory granules (Greenberg and Kolen, 1966).

In another connection, we have been interested in a compound that has both alpha- and beta-adrenergic blocking abilities, the beta-blocking activity being somewhat the greater, as well as an anticholesterolemic action. This compound has been found to prevent the induction of arrythmia by infusion of epinephrine after exposure to halocarbon compounds. Both phenoxybenzamine and propranolol also have this ability. It is apparent, therefore, that both alpha- and beta-receptors may be involved in the sensitization of the heart by halocarbon compounds to the arrhythmogenic action of extraneous epinephrine.

Moe et al. (1948) showed clearly the involvement of the peripheral alpha-receptors of the cardiovascular system in the induction of idioventricular rhythms by epinephrine administered during cyclopropane anesthesia. Kunkel et al. (1951) found that veratramine, which did not alter the pressor actions of epinephrine but did reduce its cardioaccelerator effect, was capable of preventing ventricular fibrillation induced by epinephrine in cats that had been exposed to vapors of benzene. It is possible, therefore, that effects on both inotropic and chronotropic properties of the myocradium may be involved in such phenomena as the sensitization by halocarbon compounds that we are studying. Depression of myocardial contractility without significant beta-adrenergic blocking action actually is capable of mitigating the arrhythmogenic action of daunomycin (Burka et al., 1970).

ACKNOWLEDGEMENTS

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REFERENCES

1. Bamforth, B. J., K. L. Siebecker, R. Kraemer and O. S. Orth; "Effect of Epinephrine on the Dog Heart During Methoxyflurane Anesthesia;" Anesthesiol., 22:169-173, 1961.

2. Brow, G. R., C. L. H. Long and J. Beattie; "Irregularities of the Heart under Chloroform. Their Dependence on the Sympathetic Nervous System;" J. Am. Med. Assoc., 95:715-716, 1930.

3. Burka, B., E. Herman and J. Vick; "Role of the Sympathetic Nervous System in Daunomycin - Induced Arrhythmia in the Monkey;" Brit. J. Pharmacol., 39:501-510, 1970.

4. Flacke, W. and M. H. Alper; "Actions of Halothane and Norepinephrine in the Isolated Mammalian Heart;" Anesthesiol., 23:793-801, 1962.

5. Greenberg, R. and C. A. Kolen; "Effects of Acetylcholine and Calcium Ions on the Spontaneous Release of Epinephrine from Catecholamine Granules;" Proc. Soc. Exp. Biol. Med., 121:1179-1184, 1966.

6. Hall, K. D. and F. H. Norris, Jr.; "Fluothane Sensitization of Dog Heart to Action of Epinephrine;" Anesthesiol., 19:631-641, 1958.

7. Katz, R. L., R. S. Matteo and E. M. Papper; "The Injection of Epinephrine during General Anesthesia with Halogenated Hydrocarbons and Cyclopropane in Man. 2. Halothane;" Anesthesiol., 23:597-600, 1962.

8. Kunkel, A. M., A. H. Oikemus, B. P. McNamara and J. H. Wills; Acetylcholine-Induced Ventricular Fibrillation; MLRR No. 63, June 1951.

9. Langer, G. A. and S. D. Serena; "Effects of Strophanthidin Upon Contraction and Ionic Exchange in Rabbit Ventricular Myocardium: Relation to Control of Active State;" J. Mol. Cell. Cardiol., 1:65-90, 1970.

10. Levy, A. G. and T. Lewis; "Heart Irregularities, Resulting from the Inhalation of Low Percentages of Chloroform Vapour, and their Relationship to Ventricular Fibrillation;" Heart, 3:99-112, 1911.

11. Lund, A.; "Simultaneous Fluorimetric Determination of Adrenaline and Noradrenaline in Blood;" Acta Pharmacol. Toxicol., 6:137-146, 1950.

12. Mars, H. and J. Krall; "L-DOPA and Cardiac Arrhythmias;" New Engl. J. Med., 285:1437

13. McNamara, B. P., E. A. Fine and J. H. Wills; Ventricular Fibrillation Induced by Epinephrine during Asphyxia; MLRR No. 129, August 1952.

14. Moe, G. K., S. D. Malton, B. R. Renick and W. A. Freyburger; "The Role of Arterial Pressure in the Induction of Idioventricular Rhythms under Cyclopropane Anesthesia;" J. Pharmacol. Exp. Therap., 94:319-327, 1948.

15. Morris, L. E., M. H. Noltensmeyer and J. M. White, Jr.; "Epinephrine-Induced Cardiac Irregularities in the Dog during Anesthesia with Trichloroethylene, Cyclopropane, Ethyl Chloride, and Chloroform;" Anesthesiol. 14:153-158, 1953.

16. Raventos, J.; "The Action of Fluothane-a New Volatile Anesthetic;" Brit. J. Pharmacol., 11;394-409, 1956.

17. Reinhardt, C. F., A. Azar, M. E. Maxfield, P. E. Smith, Jr. and L. S. Mullen; "Cardiac Arrhythmias and 'Aerosol Sniffing';" Arch. Env. Hlth., 22:265-279, 1971.

18. Seller, R. H.; "The Role of Magnesium in Digitalis Toxicity;" Am. Heart J., 82:551-556, 1971.

19. Taylor, G. J. and W. S. Harris; "Cardiac Toxicity of Aerosol Propellants;" J. Am. Med. Assoc., 214:81-85, 1970.

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PAPER NO. 10

THE EFFECTS OF FREON® 11 AND CERTAIN DRUGS ON ISOLATED AURICLES

J. H. Wills
D. Silber

Albany Medical College
Albany, New York

Last year, I reported that 6 fluorinated halocarbon compounds that we had studied, Freons® 11, 12, 116, and C-318 and Halons® 1301 and 2402, had been found to sensitize the myocardium to the arrhythmogenic action of epinephrine. As an extension of that work, we undertook a study of the effect of Freon® 11, one of the most potent sensitizing compounds, on isolated atrial tissue.

We used the auricles of the heart of the rat, which were suspended between the bendable member of a bonded strain-gauge transducer and a fixed point and immersed in a bath of Locke's solution. This bath was aerated through a fritted glass disk with a gas mixture containing 95% O_2 and 5% CO_2 pumped by a diaphragm pump. The same pump was arranged to transfer the same mixture of gases containing a small amount of vaporized Freon® 11 from a spirometer through the organ bath. The set-up is represented in Figure 1.

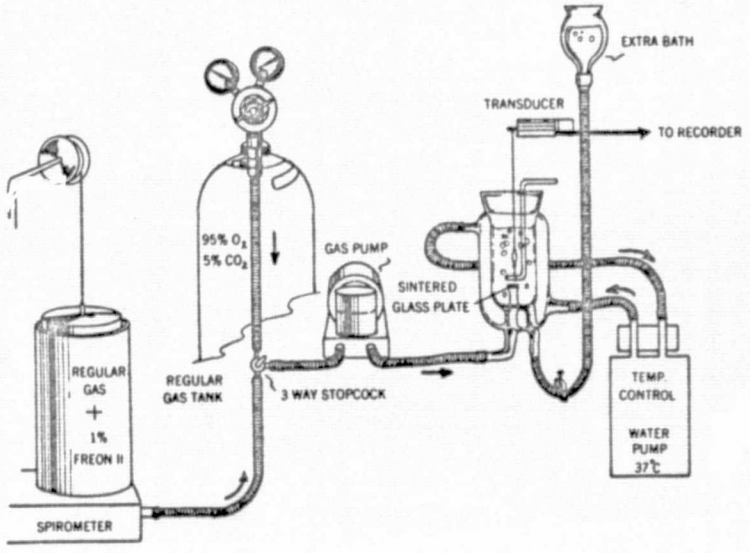

Figure 1.
Diagram of Apparatus

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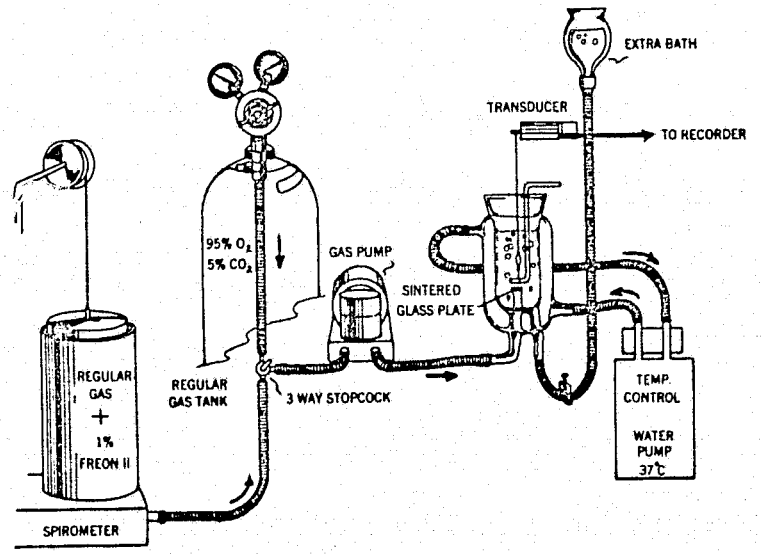

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Figure 2 shows the beginning of an exposure of a pair of auricles to the gas mixture containing 1% (v/v) of vaporized Freon® 11. It is obvious that there was no immediate effect After a period of exposure of the auricles to the gas mixture containing Freon® 11, there was a marked decrease in their rate of repetitive contraction (Figure 3). This was followed fairly promptly by complete cessation of contraction (Figure 4).

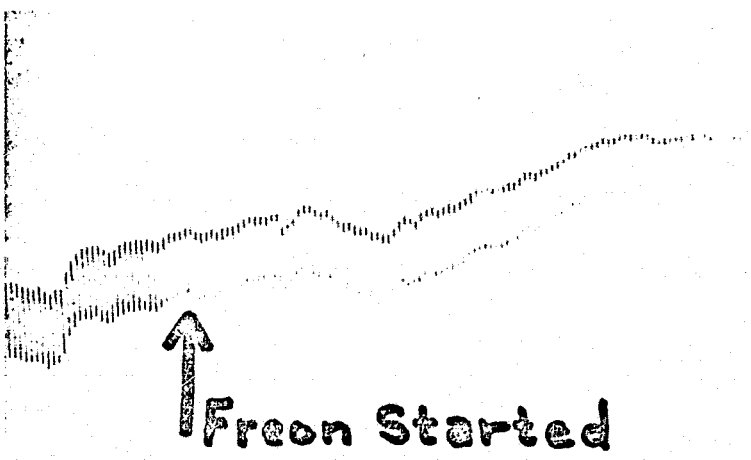


Figure 2. Beginning Exposure of Auricles to Aerating Gas Containing Freon® 11.

Upon replacement of the gas mixture containing Freon® 11 by the uncontaminated gas, rhythmic contraction by the auricles was resumed rapidly (Figure 5) and continued for long periods of time thereafter. This recovery of normal rhythmic contraction indicates to us that the exposure to vaporized Freon® 11, even though it was pressed to the point of stopping the spontaneous contractions of the auricles, did not injure the auricular muscles.

Figure 3. Decrease in Rate of Beating of Auricles Exposed to Vaporized Freon® 11.

5

Figure 4. Cessation of Contraction of Auricles Exposed to Vaporized Freon® 11.

7

Figure 5. Resumption of Beating by Auricles after Aeration with Gas Containing no Freon® 11.

Last year, we presented evidence that the sensitization of the heart to extrinsic catecholamines by inhalation of vapors of Freon® 11 was not related to an increased concentration of norepinephrine within the myocardium, to enhanced formation of either DOPA or DOPamine, or to altered permeability of the myocardial cells to K. Because of the well-known ability of calcium to link the excitatory and contractile processes in the myocardial muscle (Langer, 1968; Bailey and Dresel, 1968; Little and Sleator, 1969; Sanborn and Langer, 1970; Saari and Johnson, 1971; Shine, Serena, and Langer, 1971), we decided to use the isolated auricles to study the effect of a low external concentration of calcium on the response of the heart to catecholamines as well as on spontaneous activity by the auricles. We found that lowering the concentration of calcium in the Locke's solution to 1/10 its usual value decreased the force of contraction of the auricles with only a slight, immediate effect to decrease their rate of beating, but eventually did stop the contractions; the auricles did not respond to addition of epinephrine to the fluid within the bath either while they continued to beat or after they became quiescent (Figure 6). Addition of $CaCl_2$ to the fluid within the tissue bath restored both the force of contraction of the auricles (Figure 7) and their responsiveness to added catecholamines (Figure 8). The threshold concentration of calcium in the Locke's solution to render the auricles responsive to epinephrine was found to be about 0.225 of the usual concentration (or about 2.7 mg% instead of the standard 12 mg%).

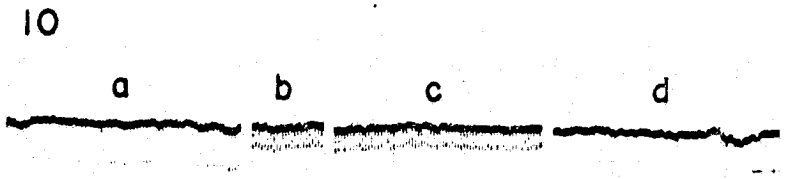


Figure 6. Decrease in force of contraction and eventual cessation of beating of auricles suspended in Locke's solution containing only 1/10 the usual concentration of Ca; lack of responsiveness of these auricles to epinephrine.

a = normal

b = after 0.2 normal Ca^{++}

c = after epinephrine

d = later, second addition of epinephrine

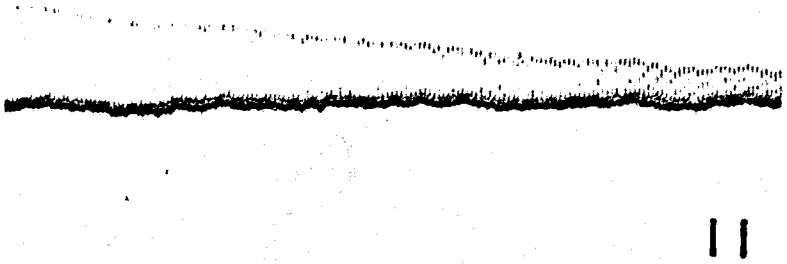


Figure 7. Restoration of contractile force by addition of $CaCl_2$ to the bath.

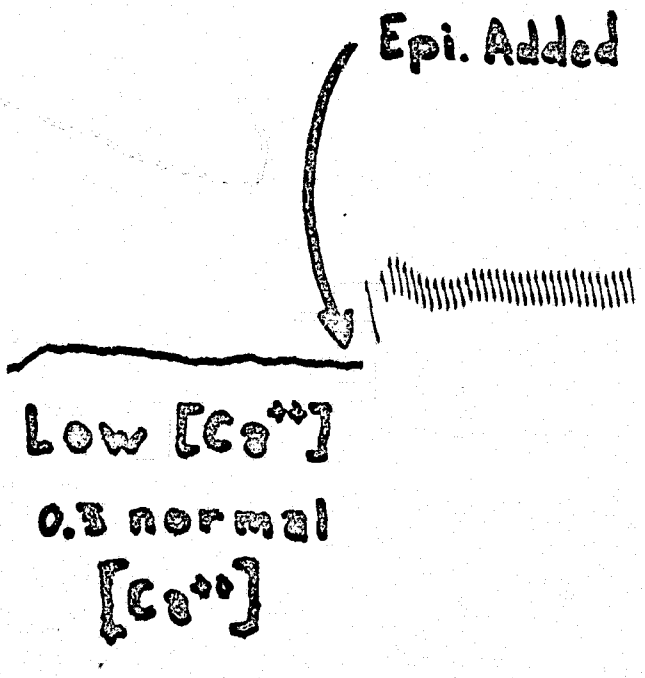


Figure 8. Restoration of responsiveness to epinephrine after addition of $CaCl_2$ to the bath.

Because we were interested in providing to NASA, if possible, guidance on ways for preventing the sensitization of the heart by the fluorinated halocarbon compounds, we thought that Verapamil (or Iproveratril or Isoptin) might be of use. When we applied this drug, which is held by some to act by preventing the migration of calcium through the excited membrane of the muscle fiber to the contractile elements, minimizing thereby the activation of ca-dependent ATPase (Fleckenstein et al., 1967; Fleckenstein, 1970), we found that its addition to the bath in a concentration of 0.48 mg/100 ml had effects quite different from those of lowered external concentration of calcium. Instead of decreasing the force of contraction, as did a low external concentration of calcium, Verapamil increased the force of contraction, but did slow the rate of beating somewhat (Figure 9.) In this figure, the rate had been decreased by only about 7%. With longer exposure to the drug, the rate fell further (to about 33% of its original value), but the force of contraction increased even more (Figure 10).

Figure 9. Decrease in rate of beating and increase in force of contraction of auricles by addition of Verapamil to the bath.

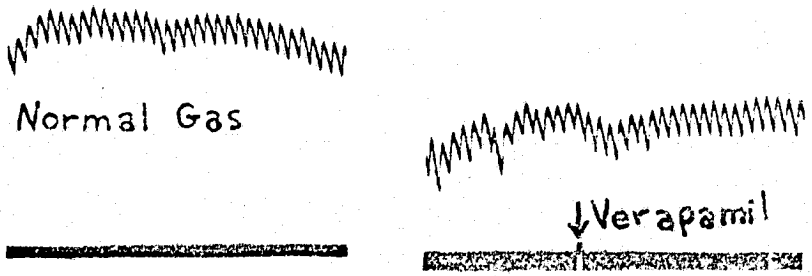
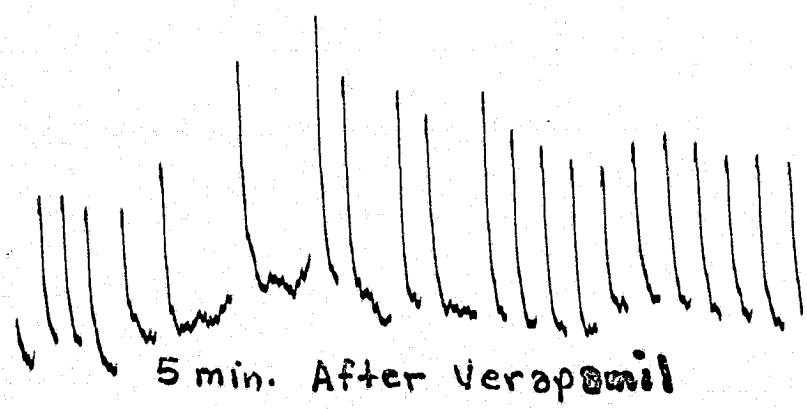


Figure 10. Further slowing and increase in force of contraction of auricles by longer contact with Verapamil.



Another drug that we have studied with our isolation auricle preparation is Na capobenate. This drug had been found to prevent the induction by epinephrine of arrhythmias in anesthetized dogs, cats and guinea pigs that were breathing vapors of halocarbon compounds. This compound, added to the bath after periods of exposure to Freon® and of recovery thereafter, induced an apparent increase in the strength of contraction of the auricular muscle (Figure 11).

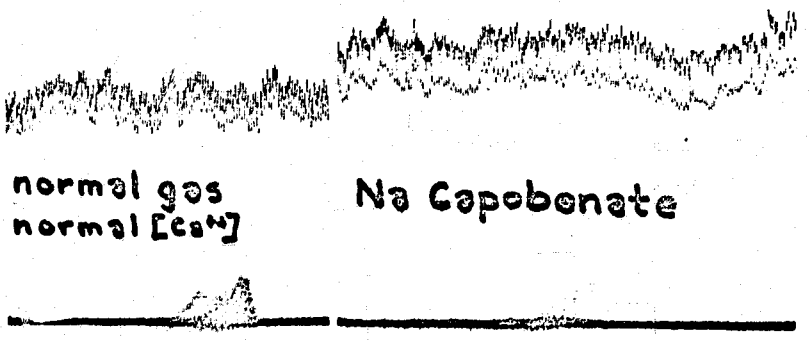


Figure 11. Increase in strength of contraction of auricles by Na capobenate added to the bath after periods of exposure to Freon® 11 and of recovery therefrom.

From this work, we derive the following conclusions:

1. Vaporized Freon® 11 apparently has enough solubility in water to be able to affect the contractile properties of auricular muscle, decreasing the rate of beating after an initial heightening of tension development.

2. Exposure to vaporized Freon® 11, although it may stop spontaneous beating of the auricles, seems not to damage the tissue, so that spontaneous beating will reappear when the Freon® has been washed out of the tissue.

3. A lowered external concentration of calcium and addition of Verapamil to the Locke's solution in which the auricles were suspended had quite different effects on the beating of the auricles, so that we tend to be somewhat skeptical about the theory that Verapamil operates entirely through preventing the ingress of calcium to the cardiac muscle cell.

4. A compound that has β-adrenergic blocking activity and also some α-adrenergic blocking activity, Na capobenate, seems to be able to improve tension development by auricular muscle after a period of exposure to vaporized Freon 11.

REFERENCES

Bailey, L. E., and P. E. Dresel, "Correlation of Contractile Force with a Calcium Pool in the Isolated Cat Heart," J. Gen. Physiol., 52:969-982, 1968.

Fleckenstein, A., "Die Zugelung des Myocardstoffwechsels durch Verapamil: Angriffspunkte und Anwendungsmoglichkeiten," Arzneimittelf, 20:1317-1322, 1970.

Fleckenstein, A., H. Kammermeir, H. J. Doring, and H. J. Freund, "Zum Wirkungsmechanismus neuartiger Koronardilatatoren mit gleichzeitig Sauerstoffeinsparenden Myokard-Effekten, Prenylamin und Iproveratril," Ztschr. Kreislauf, 56:716-744, and 839-858, 1967.

Langer, G. A., "Ion Fluxes in Cardiac Excitation and Contraction and their Relation to Myocardial Contractility," Physiol. Rev., 48:708-757, 1968.

Little, G. R., and W. W. Sleator, "Calcium Exchange and Contraction Strength of Guinea Pig Atrium in Normal and Hypertonic Media," J. Gen. Physiol., 54:494-511, 1969.

Saari, J. T., and J. A. Johnson, "Decay of Calcium Content and Contractile Force in the Rabbit Heart," Am. J. Physiol., 221:1572-1575, 1971.

Sanborn, W. G., and G. A. Langer, "Specific Uncoupling of Excitation and Contraction in Mammalian Cardiac Tissue by Lanthanum: Kinetic Studies," J. Gen. Physiol., 56:191-217, 1970.

Shine, K. I., S. D. Serena, and G. A. Langer, "Kinetic Localization of Contractile Calcium in Rabbit Myocardium," Am. J. Physiol., 221:1408-1417, 1971.

DISCUSSION

DR. STOKINGER (National Institute of Occupational Safety and Health): What's the chemical nature of those drugs? Are they amines or what?

DR. WILLS (Albany Medical College): Sodium capobenate is a fairly long chain fatty acid ester. Verapamil has an amine structure, but I can't give you its constitution in detail now. I have it in my briefcase.

DR. BACK (Aerospace Medical Research Laboratory): I looked it up. It's a dimethoxy compound that would look like a steroid if it were bent around. It has 2 benzene rings on it with 2 methoxy groups on each ring. If you could bend it around, it would look like a veratrum alkaloid but it isn't. It's in the Merck Index. I never heard of the compound before, frankly. I think it's a German drug, but I don't know whether it's ever been marketed. I'm really not familiar with it.

MR. VERNOT (University of California, Irvine): Dr. Wills, was it your intention to use this preparation as an in vitro model of the heart to try to obtain some insight into the arrythmic potential of these freons in the intact animal?

DR. WILLS: That's right. Unfortunately, however, we've not been able to demonstrate sensitization with this preparation.

MR. VERNOT: You didn't show that epinephrine, itself, had any action on the system, did you?

DR. WILLS: Yes, it does but we haven't been able to show that freon increases its action.

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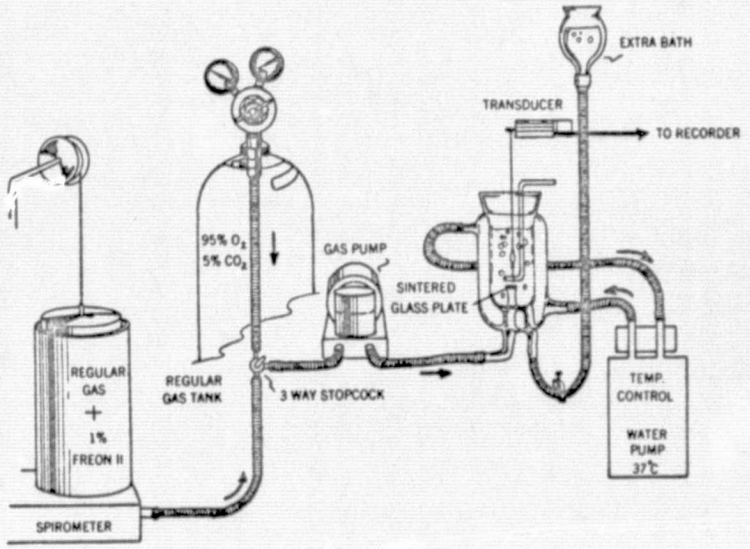

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Diagram of Apparatus

Figure 2 shows the beginning of an exposure of a pair of auricles to the gas mixture containing 1% (v/v) of vaporized Freon® 11. It is obvious that there was no immediate effect After a period of exposure of the auricles to the gas mixture containing Freon® 11, there was a marked decrease in their rate of repetitive contraction (Figure 3). This was followed fairly promptly by complete cessation of contraction (Figure 4).

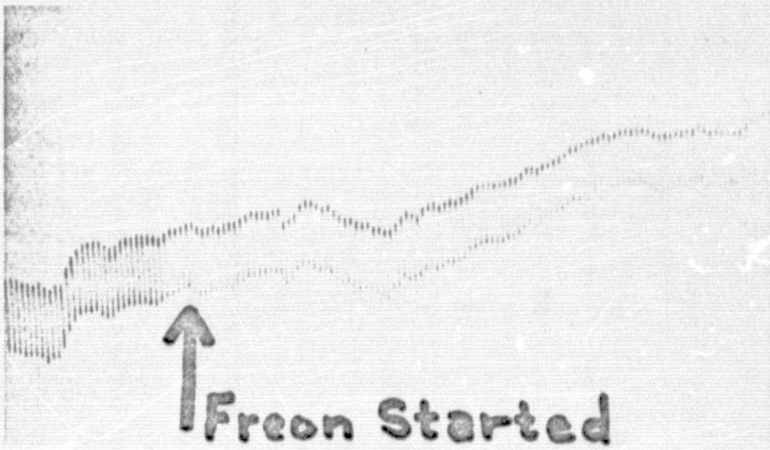

Figure 2. Beginning Exposure of Auricles to Aerating Gas Containing Freon® 11.

Upon replacement of the gas mixture containing Freon® 11 by the uncontaminated gas, rhythmic contraction by the auricles was resumed rapidly (Figure 5) and continued for long periods of time thereafter. This recovery of normal rhythmic contraction indicates to us that the exposure to vaporized Freon® 11, even though it was pressed to the point of stopping the spontaneous contractions of the auricles, did not injure the auricular muscles.

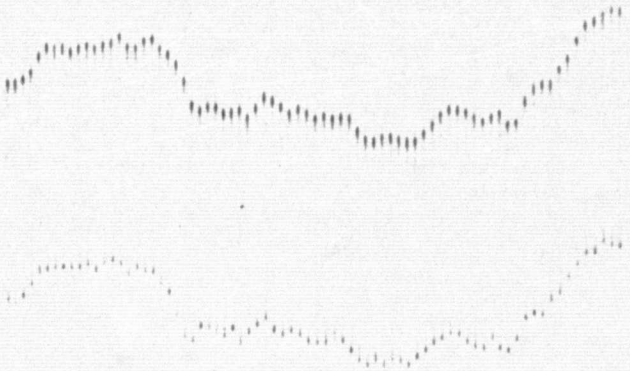

Figure 3. Decrease in Rate of Beating of Auricles Exposed to Vaporized Freon® 11.

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Figure 4. Cessation of Contraction of Auricles Exposed to Vaporized Freon® 11.

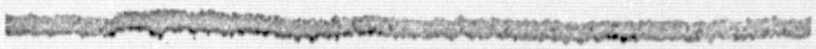

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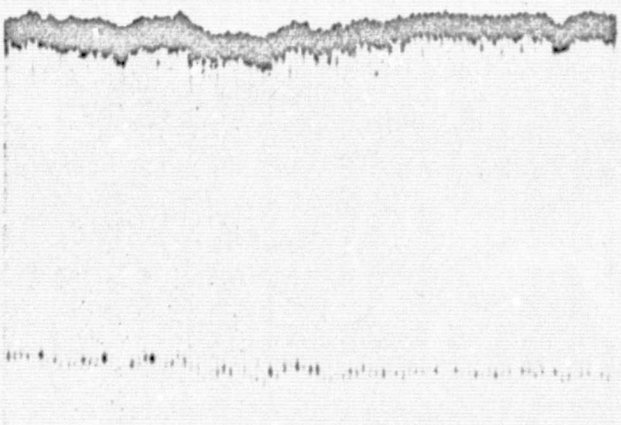
Figure 5. Resumption of Beating by Auricles after Aeration with Gas Containing no Freon® 11.



Last year, we presented evidence that the sensitization of the heart to extrinsic catecholamines by inhalation of vapors of Freon® 11 was not related to an increased concentration of norepinephrine within the myocardium, to enhanced formation of either DOPA or DOPamine, or to altered permeability of the myocardial cells to K. Because of the well-known ability of calcium to link the excitatory and contractile processes in the myocardial muscle (Langer, 1968; Bailey and Dresel, 1968; Little and Sleator, 1969; Sanborn and Langer, 1970; Saari and Johnson, 1971; Shine, Serena, and Langer, 1971), we decided to use the isolated auricles to study the effect of a low external concentration of calcium on the response of the heart to catecholamines as well as on spontaneous activity by the auricles. We found that lowering the concentration of calcium in the Locke's solution to 1/10 its usual value decreased the force of contraction of the auricles with only a slight, immediate effect to decrease their rate of beating, but eventually did stop the contractions; the auricles did not respond to addition of epinephrine to the fluid within the bath either while they continued to beat or after they became quiescent (Figure 6). Addition of $CaCl_2$ to the fluid within the tissue bath restored both the force of contraction of the auricles (Figure 7) and their responsiveness to added catecholamines (Figure 8). The threshold concentration of calcium in the Locke's solution to render the auricles responsive to epinephrine was found to be about 0.225 of the usual concentration (or about 2.7 mg% instead of the standard 12 mg%).

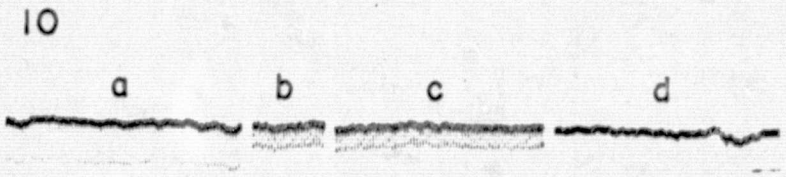


Figure 6. Decrease in force of contraction and eventual cessation of beating of auricles suspended in Locke's solution containing only 1/10 the usual concentration of Ca; lack of responsiveness of these auricles to epinephrine.

a = normal

b = after 0.2 normal Ca^{++}

c = after epinephrine

d = later, second addition of epinephrine

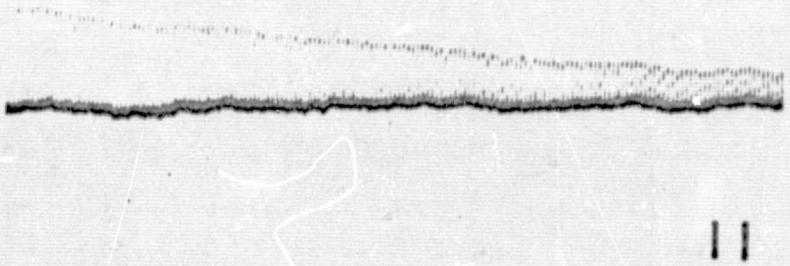


Figure 7. Restoration of contractile force by addition of $CaCl_2$ to the bath.

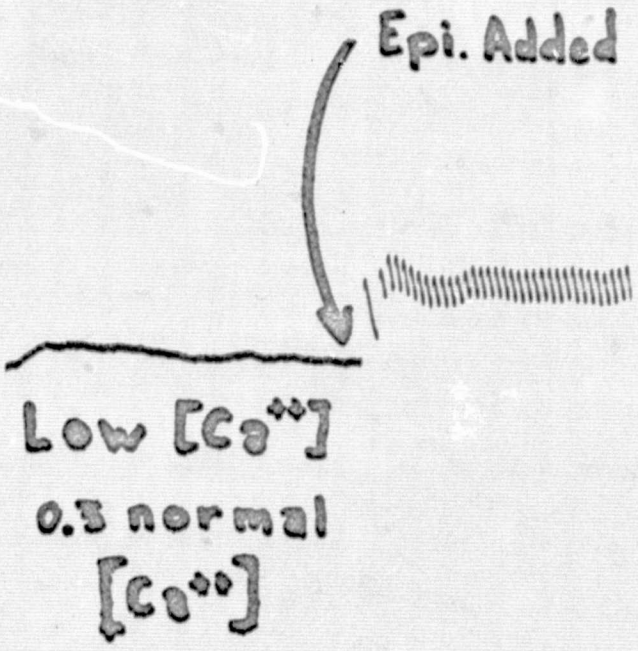


Figure 8. Restoration of responsiveness to epinephrine after addition of $CaCl_2$ to the bath.

Because we were interested in providing to NASA, if possible, guidance on ways for preventing the sensitization of the heart by the fluorinated halocarbon compounds, we thought that Verapamil (or Iproveratril or Isoptin) might be of use. When we applied this drug, which is held by some to act by preventing the migration of calcium through the excited membrane of the muscle fiber to the contractile elements, minimizing thereby the activation of ca-dependent ATPase (Fleckenstein et al., 1967; Fleckenstein, 1970), we found that its addition to the bath in a concentration of 0.48 mg/100 ml had effects quite different from those of lowered external concentration of calcium. Instead of decreasing the force of contraction, as did a low external concentration of calcium, Verapamil increased the force of contraction, but did slow the rate of beating somewhat (Figure 9.) In this figure, the rate had been decreased by only about 7%. With longer exposure to the drug, the rate fell further (to about 33% of its original value), but the force of contraction increased even more (Figure 10).

Figure 9. Decrease in rate of beating and increase in force of contraction of auricles by addition of Verapamil to the bath.

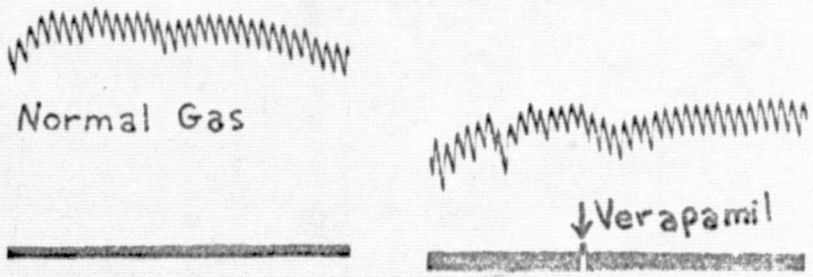
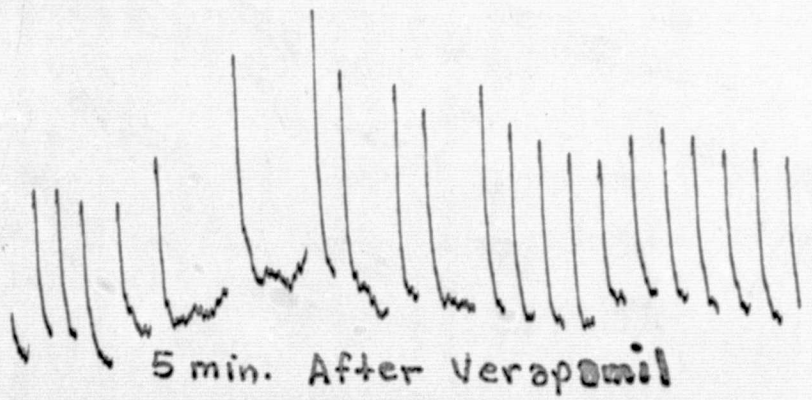


Figure 10. Further slowing and increase in force of contraction of auricles by longer contact with Verapamil.



Another drug that we have studied with our isolation auricle preparation is Na capobenate. This drug had been found to prevent the induction by epinephrine of arrhythmias in anesthetized dogs, cats and guinea pigs that were breathing vapors of halocarbon compounds. This compound, added to the bath after periods of exposure to Freon® and of recovery thereafter, induced an apparent increase in the strength of contraction of the auricular muscle (Figure 11).

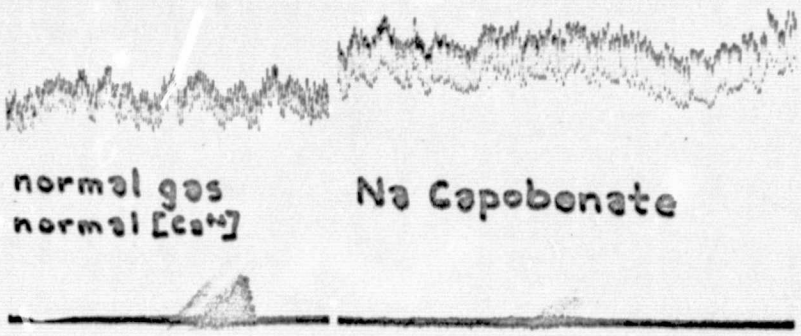


Figure 11. Increase in strength of contraction of auricles by Na capobenate added to the bath after periods of exposure to Freon® 11 and of recovery therefrom.

From this work, we derive the following conclusions:

1. Vaporized Freon® 11 apparently has enough solubility in water to be able to affect the contractile properties of auricular muscle, decreasing the rate of beating after an initial heightening of tension development.

2. Exposure to vaporized Freon® 11, although it may stop spontaneous beating of the auricles, seems not to damage the tissue, so that spontaneous beating will reappear when the Freon® has been washed out of the tissue.

3. A lowered external concentration of calcium and addition of Verapamil to the Locke's solution in which the auricles were suspended had quite different effects on the beating of the auricles, so that we tend to be somewhat skeptical about the theory that Verapamil operates entirely through preventing the ingress of calcium to the cardiac muscle cell.

4. A compound that has β-adrenergic blocking activity and also some α-adrenergic blocking activity, Na capobenate, seems to be able to improve tension development by auricular muscle after a period of exposure to vaporized Freon 11.

REFERENCES

Bailey, L. E., and P. E. Dresel, "Correlation of Contractile Force with a Calcium Pool in the Isolated Cat Heart," J. Gen. Physiol., 52:969-982, 1968.

Fleckenstein, A., "Die Zugelung des Myocardstoffwechsels durch Verapamil: Angriffspunkte und Anwendungsmoglichkeiten," Arzneimittelf, 20:1317-1322, 1970.

Fleckenstein, A., H. Kammermeir, H. J. Doring, and H. J. Freund, "Zum Wirkungsmechanismus neuartiger Koronardilatatoren mit gleichzeitig Sauerstoffeinsparenden Myokard-Effekten, Prenylamin und Iproveratril," Ztschr. Kreislauf, 56:716-744, and 839-858, 1967.

Langer, G. A., "Ion Fluxes in Cardiac Excitation and Contraction and their Relation to Myocardial Contractility," Physiol. Rev., 48:708-757, 1968.

Little, G. R., and W. W. Sleator, "Calcium Exchange and Contraction Strength of Guinea Pig Atrium in Normal and Hypertonic Media," J. Gen. Physiol., 54:494-511, 1969.

Saari, J. T., and J. A. Johnson, "Decay of Calcium Content and Contractile Force in the Rabbit Heart," Am. J. Physiol., 221:1572-1575, 1971.

Sanborn, W. G., and G. A. Langer, "Specific Uncoupling of Excitation and Contraction in Mammalian Cardiac Tissue by Lanthanum: Kinetic Studies," J. Gen. Physiol., 56:191-217, 1970.

Shine, K. I., S. D. Serena, and G. A. Langer, "Kinetic Localization of Contractile Calcium in Rabbit Myocardium," Am. J. Physiol., 221:1408-1417, 1971.

DISCUSSION

DR. STOKINGER (National Institute of Occupational Safety and Health): What's the chemical nature of those drugs? Are they amines or what?

DR. WILLS (Albany Medical College): Sodium capobenate is a fairly long chain fatty acid ester. Verapamil has an amine structure, but I can't give you its constitution in detail now. I have it in my briefcase.

DR. BACK (Aerospace Medical Research Laboratory): I looked it up. It's a dimethoxy compound that would look like a steroid if it were bent around. It has 2 benzene rings on it with 2 methoxy groups on each ring. If you could bend it around, it would look like a veratrum alkaloid but it isn't. It's in the Merck Index. I never heard of the compound before, frankly. I think it's a German drug, but I don't know whether it's ever been marketed. I'm really not familiar with it.

MR. VERNOT (University of California, Irvine): Dr. Wills, was it your intention to use this preparation as an in vitro model of the heart to try to obtain some insight into the arrythmic potential of these freons in the intact animal?

DR. WILLS: That's right. Unfortunately, however, we've not been able to demonstrate sensitization with this preparation.

MR. VERNOT: You didn't show that epinephrine, itself, had any action on the system, did you?

DR. WILLS: Yes, it does but we haven't been able to show that freon increases its action.